FROM THE INSTITUTE OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

EFFECT OF CHLORIDES OF ALKALI AND OF ALKALINE EARTH METALS ON THE ISOLATED RABBIT AURICLE

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I

EFFECT OF CHLORIDES OF ALKALI METALS ON THE ISOLATED RABBIT AURICLE

CHANGES IN THE MECHANOGRAM AND INITIAL DEFLECTION OF THE ELECTROGRAM

The presence of certain salts is of great importance for the maintenance of the rhythmicity and other properties of the heart muscle, as Ringer has already shown in his classical experiments (19—21). The electrolytes play an important role in muscular contraction in general (14). Although numerous studies have been performed since Ringer's days, the mechanism by which the different ions act on the heart seems still to be unknown. Sodium and especially potassium ion as physiological and essential cations for the normal functions of myocardium have received the attention of many authors (e.g. 1, 3, 4, 6—8, 11, 13, 15, 18, 22—24). Interest has also been shown in the effects of lithium, rubidium and cesium ions on the heart muscle (5, 14, 16, 17).

A previous study reported some observations of the effects of sodium chloride on the isotonically recorded mechanogram and electrogram of the isolated rabbit auricle (9). The present study completes the earlier investigation by systematic observations of the effects of various alkali metal chlorides. An isometrical mechanogram together with an electrogram was recorded of the isolated rabbit auricle in Locke's solution before and after the addition of repeated doses of the salts in question.

METHODS

The experiments were performed on right auricular preparations isolated from eight adult rabbits. The auricle beated spontaneously in oxygenated Locke's solution at 30°C. The mechanogram was recorded isometrically with the aid of a piezoelectrical crystal under a constant stretch of 3 mm throughout the test series. The electrogram was recorded with a »Triplex»-electrocardiograph. The non-polarizable silver-silver chloride electrodes used were prepared from silver wire insulated with shellac. The electrodes were in direct contact with the preparation in Locke's solution. The technical details of the isolation of the auricle and the recording system have been published previously (9).

Lithium, sodium, potassium, rubidium and cesium, which are members of the alkali series of metals in the periodic chart of the elements, were added as chlorides to the Locke's solution surrounding the preparation. The recording was made 4 to 5 minutes after the addition of the salts. The procedure was repeated until signs of severe impairment in contraction force and/or rate appeared. The average duration of the various series was as follows: lithium 16 min. (range 12 to 19 min.), sodium 18 (15 to 23) min., potassium 15 (10 to 20) min., rubidium 12½ (8 to 17) min. and cesium 19½ (15 to 23) min. Immediately after the last recording, the preparation was washed two to three times with Locke's solution, and a new test series was started after a pause of 10 to 30 minutes or even more, when the normal functions were stabilized.

From the recorded tracings the following properties were measured: cycle length, duration of contraction and relaxation phases, contraction force, R-wave height and duration of the QRS interval. The initial observation before addition of the chloride served as a control. In the case of the contraction force and amplitude of the R wave, the value of the initial observation was taken as the unit with a value of 100 in each test series. This made it possible to note without additional calculation the relative changes in these properties caused by addition of the salts in question. Alterations in the T wave were not analyzed because of the great variations in its behaviour. A much bigger material is needed to draw reliable conclusions in this respect. It was possible to perform all the tests with the various chlorides one after another with the

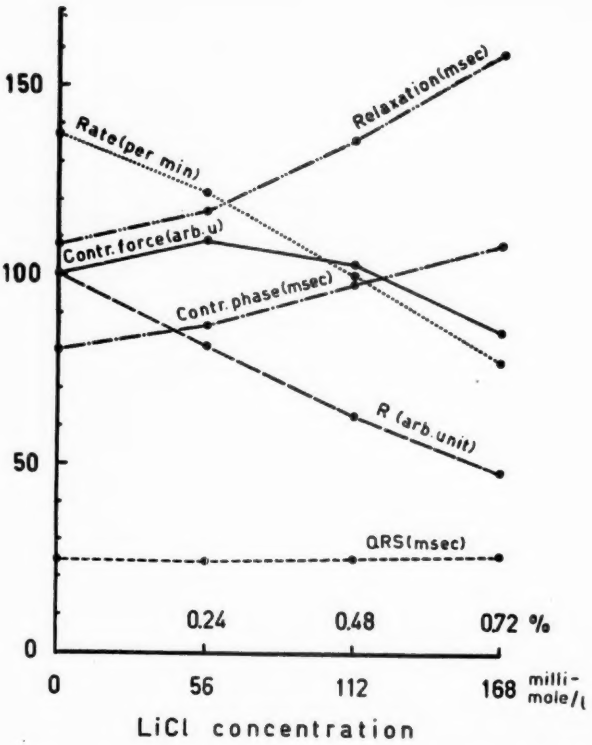

Fig. 1. — Effect of lithium chloride on the isolated rabbit auricle. Changes in the mechanogram and initial deflection of the electrogram.

same preparation. When repeated, the results of two series with the same substance on the same preparation gave similar results. With every salt, 8 to 9 series were recorded from 6 to 8 preparations. Fechner's formula (2) and Table IV in Kemp's book (12) were used in the statistical treatment of the results.

RESULTS

The results of the various tests are presented graphically as mean values in Figures 1 to 5. Table 1 complements the figures as it shows the statistical significance of the changes in the extreme conditions.

Lithium Chloride. — Addition of lithium chloride slowed the beating rate (Fig. 1). The contraction force showed small altera-

TABLE 1

EFFECT OF ALKALI METAL CHLORIDES ON FUNCTIONS OF THE ISOLATED RABBIT AURICLE. RESULTS RECORDED UNDER BASIC CONDITIONS AND IN THE HIGHEST SALT CONCENTRATIONS TO WHICH THE PREPARATION WAS EXPOSED

	Cation	Initial Recording	Final Recording	Difference	Standard Error of Difference	Diff./S.E.	Probability
Contraction Force (arb. unit)	Li	100	85	—15	10	1.5	> 0.1
	Na	100	84	—16	5	3.2	< 0.02
	K	100	31	—69	6	10.2	< 0.001
	Rb	100	34	—66	11	6.0	< 0.001
	Cs	100	48	—52	11	4.7	< 0.01
Rate of Beat (per minute)	Li	137	77	—60	17	3.5	< 0.01
	Na	131	125	—6	9	0.7	> 0.1
	K	122	64	—58	13	4.5	< 0.01
	Rb	132	55	—77	19	4.1	< 0.01
	Cs	117	68	—49	12	4.1	< 0.01
Contraction Phase (msec)	Li	80	108	+28	6	4.7	< 0.01
	Na	84	98	+14	3	4.7	< 0.01
	K	89	99	+10	6	1.7	> 0.1
	Rb	87	111	+24	5	4.8	< 0.01
	Cs	93	107	+14	5	2.8	< 0.05
Relaxation Phase (msec)	Li	108	159	+51	7	7.3	< 0.001
	Na	112	150	+38	8	4.8	< 0.01
	K	116	134	+18	9	2.0	< 0.1
	Rb	111	145	+34	9	3.8	< 0.01
	Cs	115	141	+26	11	2.3	< 0.1
R (arb. unit)	Li	100	48	—52	8	6.5	< 0.001
	Na	100	52	—48	6	8.0	< 0.001
	K	100	46	—54	14	3.9	< 0.01
	Rb	100	29	—71	9	7.9	< 0.001
	Cs	100	68	—32	7	4.6	< 0.01
QRS (msec)	Li	24	26	+2	2	1.0	> 0.1
	Na	28	19	—9	3	3.0	= 0.02
	K	26	74	+48	5	9.6	< 0.001
	Rb	24	72	+48	8	6.0	< 0.001
	Cs	24	70	+46	11	4.2	< 0.01

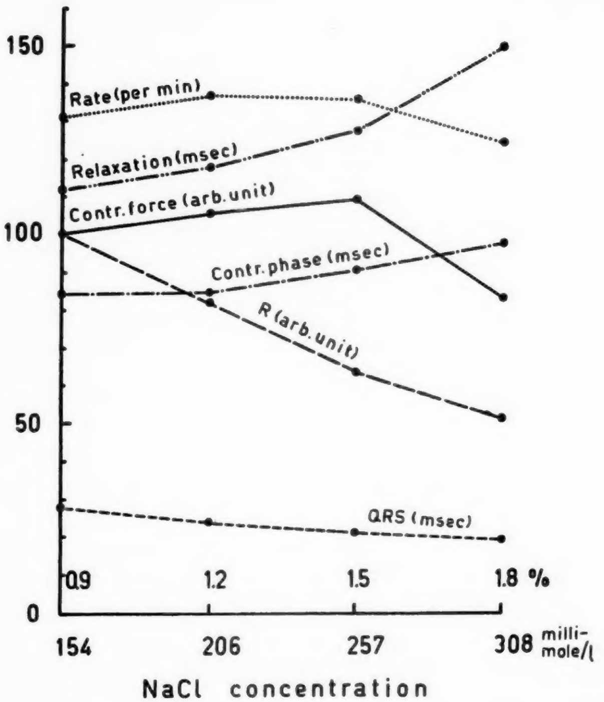

Fig. 2. — Effect of sodium chloride on the isolated rabbit auricle. Changes in the mechanogram and initial deflection of the electrogram.

tions, at first in an increasing and then in a decreasing direction. The duration of the isometrical contraction prolonged with increasing lithium concentration. This change was observed in both the contraction and the relaxation phase. The amplitude of the recorded action potential, the R wave, diminished. The QRS interval remained practically unchanged.

Sodium Chloride. — Increase in the sodium chloride concentration caused very small changes in the contraction force and rate and the duration of the various phases of isometrical contraction before the total concentration exceeded 1.5 per cent (Fig. 2). Electrographic observations showed more marked changes. When the concentration was doubled from that found in Locke's solution, the duration of both the contraction and the relaxation phase showed a significant prolongation. The amplitude of R and duration of QRS decreased more and more.

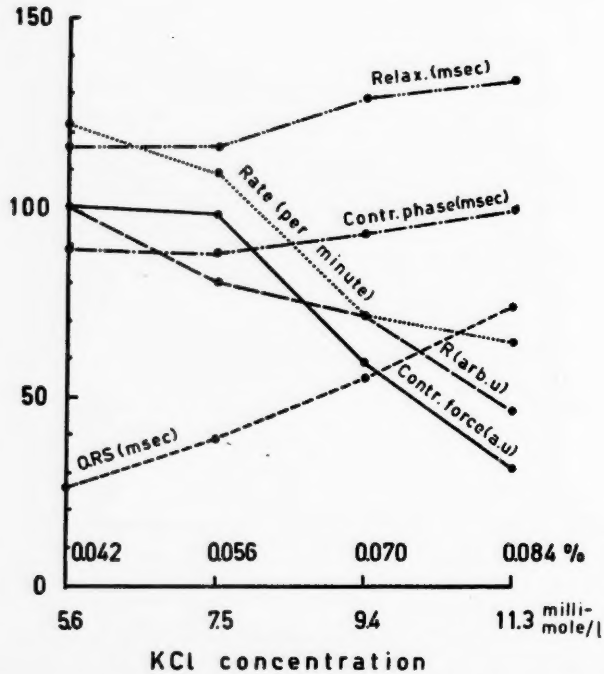

Fig. 3. — Effect of potassium chloride on the isolated rabbit auricle. Changes in the mechanogram and initial deflection of the electrogram.

Potassium Chloride. — The potassium chloride concentration was doubled from that in Locke's solution after the last addition. The mechanogram remained nearly unchanged when the potassium concentration was raised by one third, i. e. to 0.056 per cent (Fig. 3). After this the contraction force and rate showed signs of impaired activity. The changes in the duration of contraction were not significant. The diminution of the R wave and especially the prolongation of the QRS interval were noticeable. In the highest potassium chloride concentrations the form of the QRS complex was irregular and its width difficult or impossible to dertermine exactly.

Rubidium Chloride. — As a result of the addition of rubidium chloride the contraction force weakened, the rate retarded and the duration of various phases of the contraction was prolonged (Fig. 4). The electrogram showed diminution in the amplitude of R and prolongation in the QRS interval. The higher the rubidium con-

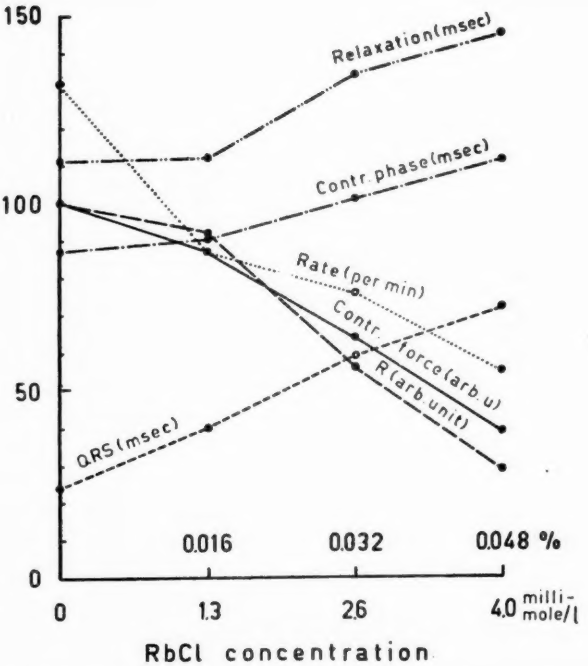

Fig. 4. — Effect of rubidium chloride on the isolated rabbit auricle. Changes in the mechanogram and initial deflection of the electrogram.

centration the more pronounced were the changes in the mechanogram and electrogram.

Cesium Chloride. — Changes similar to those found in the potassium and rubidium series were also observed after addition of cesium chloride, as Fig. 5 shows.

DISCUSSION

The results are presented as a function of the concentration of the salts in question. It must be remembered, however, that the effect depends not only on the concentration but also on the duration of the influence of the salt in any concentration. Therefore the various test series were performed according to a fairly uniform time table. There were naturally certain individual variations in various preparations. The absolute figure of concentration has less value as such than as a part of the present study as a whole.

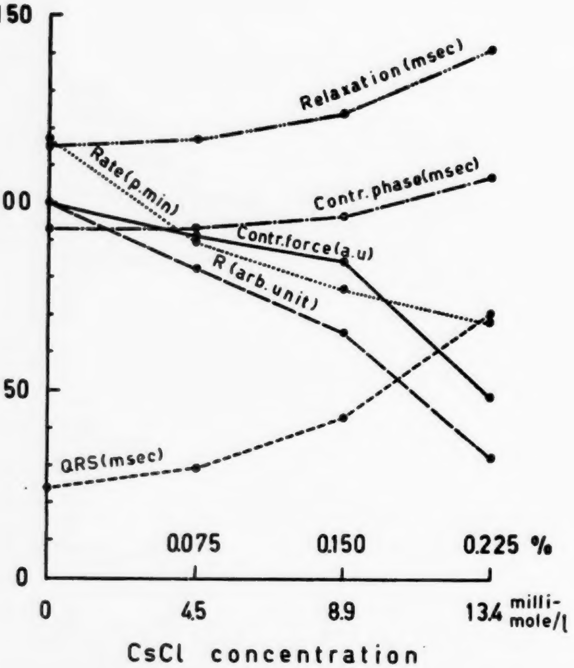

Fig. 5. — Effect of cesium chloride on the isolated rabbit auricle. Changes in the mechanogram and initial deflection of the electrogram.

Comparing the present results with previous reports shows many points of contact. Only a few studies need be mentioned. Reference is made especially to the bibliography of Lenzi's & Caniggia's monograph on the nature of myocardial contraction (14). The depressing effect of lithium chloride on the beating rate has been observed in the isolated dog and turtle heart (5). Shortening of the QRS interval was noted by Lenzi & Caniggia (14) in tortoise after the intravenous injection of lithium chloride. McKusic (17) reported an elevation of serum potassium as a result of parenteral lithium chloride administration to mammals. The QRS complex widened but this was explained on the basis of secondary changes in the ratio of intracellular and extracellular potassium concentration. Butcher *et al.* (5) found no changes in the QRS interval of an isolated heart resulting from a high lithium concentration in the perfusate. This is in accordance with the present study. The decrease in the voltage of QRS was observed by Lenzi & Caniggia (14) and Butcher *et al.* (5).

The observations of the sodium chloride addition to Locke's solution concur with those of previous studies on isolated rabbit auricle (9) and heart preparations of cold-blooded animals (1, 14).

Potassium, rubidium and cesium chloride caused, above all, prolongation of the QRS interval. This has been observed also in tortoise (14) after the intravenous injection of the cations in question, although the height of the QRS complex did not diminish. In this connection Hodgkin's (10) observation on the surface membrane of an isolated axon from *Carcinus maenas* may be of interest. He found that small changes in the external potassium concentration caused large and rapidly reversible changes in the membrane conductance. The conductance increased when the potassium concentration was elevated. Similar increases in conductance were produced by adding salts in the following molar ratios: rubidium chloride 0.8, potassium chloride 1.0, cesium chloride 2.2, sodium chloride 40 and lithium chloride 40. If the maximum concentrations used in the present study are listed according to the molar ratios, we obtain the following figures, respectively: 0.7, 1.0, 2.4, 27 and 29.5. The probable changes in the membrane conductance of the auricular preparation, however, do not alone explain the present results. For instance, the changes in the QRS interval in sodium chloride and in potassium chloride series were the opposite.

The alkali metal cations include representatives of both physiological and non-physiological ions. Their close chemical properties have many physiological correlations. The effects of lithium and sodium were very similar in the present test series. The concentration of both cations tolerated by the auricle were of the same class of magnitude. The shortening of the QRS interval was observed only in the sodium chloride tests. Lithium however, did not prolong it as the other alkali metal ions were found to do.

The effects of potassium, rubidium and cesium chloride were all very similar. Only quantitative differences were observed. The concentrations of these three salts tolerated by the preparation did not differ much and were much lower than those of lithium and sodium chlorides.

Common to the effects of the various cations used was a slight prolongation of the contraction period and diminution of the R wave. The electrolytes added to the surrounding medium of the

heart muscle act on it by increasing the osmotic pressure and conductance and by their specific ionic and molecular effects (23). The greater the number of particles per volume unit of a solution, the higher is the osmotic pressure. The greater the concentration, the better is the electrical conductivity of a solution. Although there are differences in the equivalent conductivity of various alkali metal chlorides and the degree of dissociation may vary in various test series, there is hardly any common explanation for the effects of the salts studied. The toleration of lithium and sodium chloride concentration was so high that the effect of change in osmotic pressure and conductivity must be considered. Potassium, rubidium and cesium chloride were equally effective in much lower concentrations. Therefore the specific effects on the membrane and contractile functions are to be regarded as of primary importance.

SUMMARY

The effect of lithium, sodium, potassium, rubidium and cesium chloride on the mechanogram and electrogram of the isolated rabbit auricle beating spontaneously in oxygenated Locke's solution was studied.

Lithium and sodium chloride on the one hand and potassium rubidium and cesium chloride on the other form two groups with approximately similar effects. Addition of all the salts studied resulted in the diminution of the R deflection and impairment of the mechanical activity. The duration of the contraction and relaxation phase was more or less prolonged. Only sodium chloride shortened the QRS interval. Lithium chloride did not affect it. All the other chlorides prolonged QRS markedly.

The specific effect of the various cations seems to be the most important factor which characterizes the action of alkali metal chlorides on the heart muscle.

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II

EFFECT CHLORIDES OF ALKALINE EARTHS ON THE ISOLATED RABBIT AURICLE

MECHANOGRAM AND INITIAL DEFLECTION OF THE ELECTROGRAM

Cations of the alkaline earth group are of interest as regards their influence on the heart muscle. Calcium ion is one of the essential cations in equilibrated salt solutions which are able to keep an isolated heart or a fragment of it beating for a long period. Magnesium is a constituent part of the composition of Thyrode's and Dale's solution. There are certain similarities in the physiological effects of various chemically closely related cations. The effects of five alkali metal chlorides on the isometrical mechanogram and initial deflection of the electrogram of the isolated rabbit auricle have been reported in another paper (10). The present work is a similar study with chlorides of the following alkaline earth metals: beryllium, magnesium, calcium, strontium and barium.

MATERIAL AND METHODS

Fifteen adult male rabbits were used as the experimental animals. Nine to 11 test series were performed on 6 to 10 preparations. The isolation of the auricle, the recording of the mechanogram and electrogram and some other technical details have been described in previous papers (9, 10). The various chlorides were added as 5 or 10 per cent solutions to the Locke's solution surrounding the preparation. Several recordings were made after repeated additions of the respective salts, until the mechanical functions showed signs

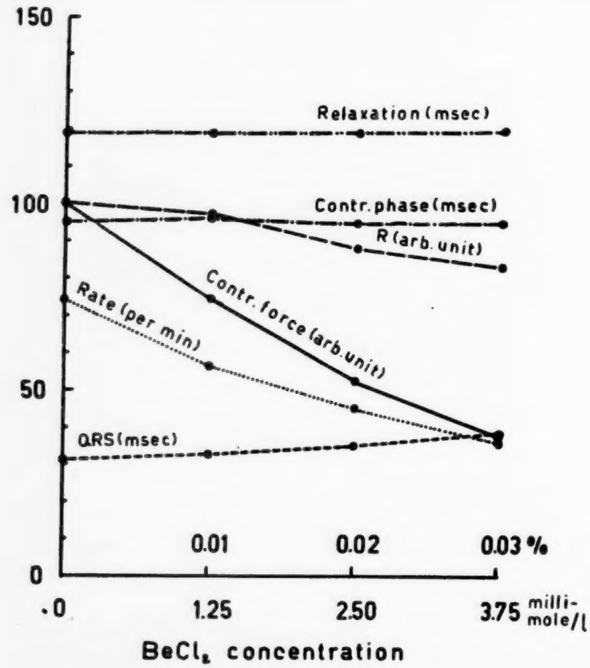

Fig. 1. — Effect of beryllium chloride on the isolated rabbit auricle. Changes in the mechanogram and initial deflection of the electrogram.

of severe impairment. In many cases the auricle suddenly stopped beating. The tolerance of the different preparations differed markedly. Thus the duration of each individual test series was generally dependent on this. The mean duration of the various test series was as follows: beryllium 11 min. (range 7 to 20 min.), magnesium 10 (4 to 14) min., calcium 26 (14 to 36) min., strontium 26 (16 to 54) min. and barium 17 (11 to 24) min.

RESULTS

The mean values of the results of the various experiments are presented graphically in Figures 1 to 5. These are complemented by Table 1 which presents the initial and final recordings and the statistical significance of their differences.

Beryllium Chloride.—Fig. 1 shows the changes caused by addition of beryllium chloride. Both the contraction force and rate were

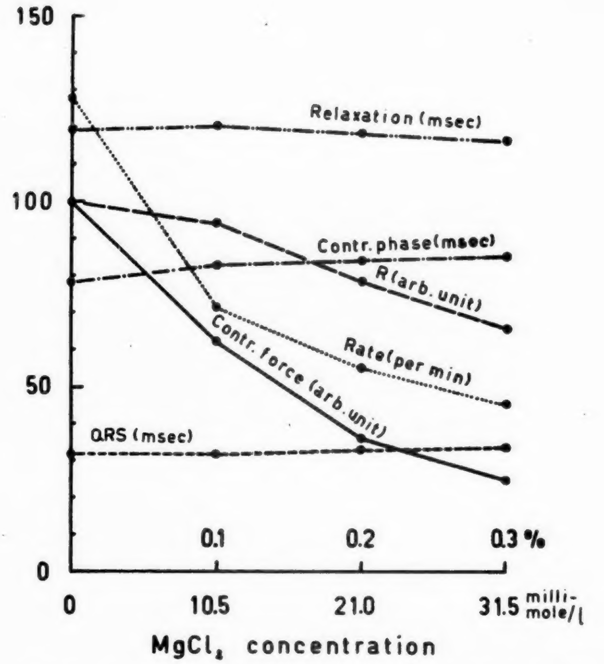

Fig. 2. — Effect of magnesium chloride on the isolated rabbit auricle. Mechanogram and initial deflection of the electrogram.

powerfully affected in a negative direction. Neither the duration of contraction phase nor that of the relaxation showed any alterations. The R wave diminished and the QRS interval was prolonged but in both cases the changes were moderate.

Magnesium Chloride. — The effects of magnesium chloride were very similar to those of beryllium chloride: weakening of the contraction force, retardation of the beating rate, lowering of the recorded amplitude of R and no changes in the duration of mechanical contraction. The QRS interval, however, remained unchanged. The magnesium chloride concentration tolerated by the preparation and which resulted in the changes described was nearly tenfold that of the beryllium chloride tests.

Calcium Chloride. — Physiologically the most important cation in the present test, calcium (Fig. 3) caused many changes which were opposite to those of the previous cations. The contraction force increased to more than three times that of the initial value.

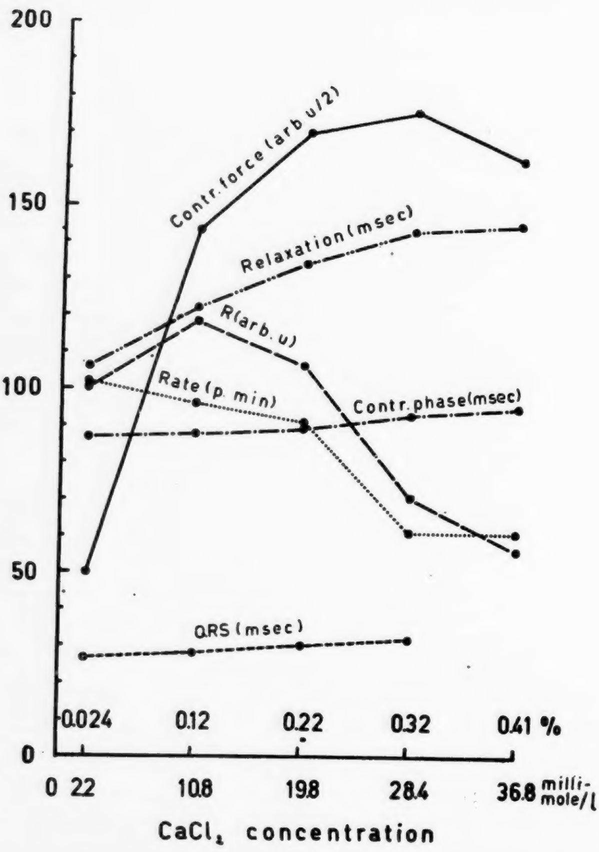

Fig. 3. — Effect of calcium chloride on the auricular preparation. Changes in the mechanogram and initial deflection of the electrogram. The curve of contraction force is based on the recorded figures divided by two.

The effect on rate was somewhat diverging. In some cases it was accelerated with a moderate dose. Temporary periods of retarded rate were often observed. On the average, retardation of the rate was established first in the highest concentrations of calcium chloride, before the preparation stopped the beating. It was possible to raise the calcium chloride concentration 10 to 30 times from that in Locke's solution before the cessation of contractions. The contraction phase remained unchanged in spite of a rise in the calcium chloride concentration. The relaxation, however, became more incomplete and prolonged. The amplitude of the R wave showed

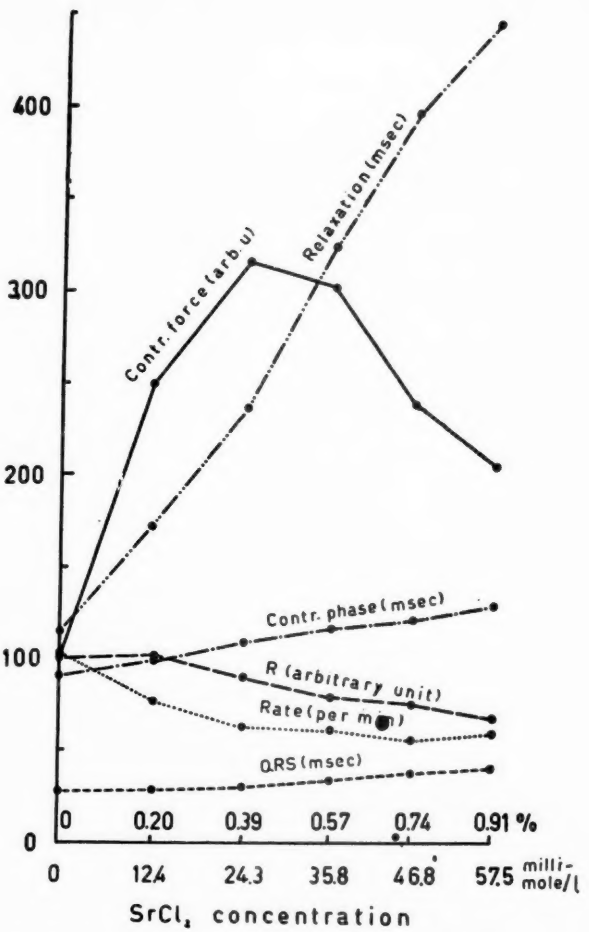

Fig. 4. — Effect of strontium chloride on the isometrical contraction and initial deflection of the electrogram of the isolated rabbit auricle. Note the scale of the ordinate compared with that in the other figures of the present study.

divergent changes after the first calcium dose. At the end of the test series it was diminished. The QRS interval showed hardly any changes.

Strontium Chloride. — The effects of strontium chloride are shown in Fig. 4. The increase in contraction force was nearly similar to that caused by calcium chloride. The rate of beat, on the contrary, retarded regularly. Both the contraction and relaxation phase of the isometrical contraction were prolonged when strontium

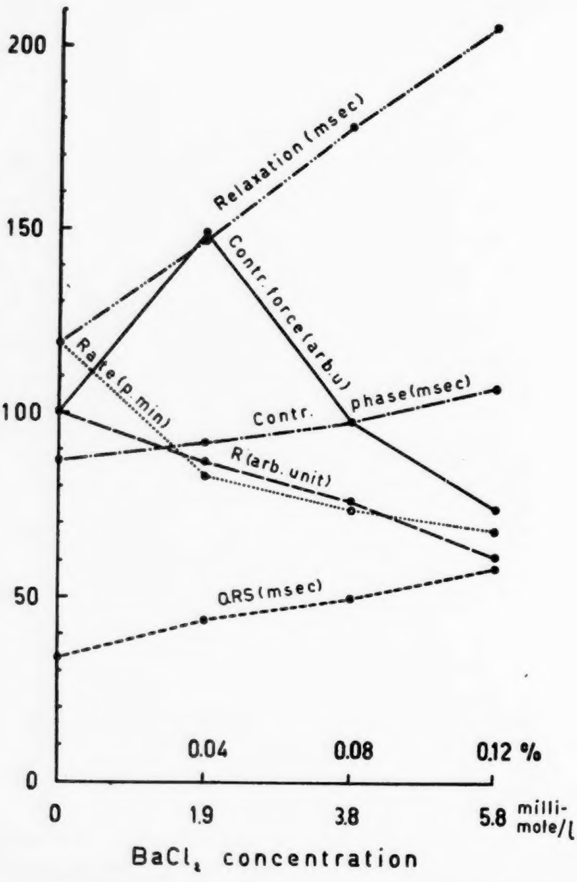

Fig. 5. — Mechanogram and electrogram recordings in various barium chloride concentrations.

chloride was added. The prolongation of relaxation was exceedingly marked. At the same time the relaxation became more and more incomplete. The R wave diminished and the QRS interval prolonged when strontium chloride was added.

Barium Chloride. — A small dose of barium chloride strengthened the contractions (Fig. 5). Later this was followed by a change in the opposite direction. The rate of beat was retarded from the very beginning. The duration of contraction and relaxation was prolonged, especially that of the latter phase. At the same time the R wave became lower and the QRS interval longer.

TABLE 1

EFFECT OF CHLORIDES OF ALKALINE EARTH METALS ON THE FUNCTIONS OF THE ISOLATED RABBIT AURICLE. RESULTS RECORDED UNDER BASIC CONDITIONS AND IN THE HIGHEST SALT CONCENTRATIONS TO WHICH THE PREPARATION WAS EXPOSED

	Cation	Initial Recording	Final Recording	Difference	Standard Error of Difference	Diff./S.E.	Probability
Contraction Force (arb. unit)	Be	100	38	—62	6	10.3	< 0.001
	Mg	100	25	—75	5	15.0	< 0.001
	Ca	100	325	+225	70	3.2	< 0.02
	Sr	100	204	+104	42	2.5	< 0.05
	Ba	100	74	—26	9	2.9	< 0.02
Rate of Beat (per minute)	Be	74	36	—38	7	5.4	< 0.001
	Mg	127	45	—82	20	4.1	< 0.01
	Ca	102	61	—41	15	2.6	< 0.05
	Sr	103	59	—44	16	2.8	< 0.05
	Ba	119	68	—51	13	3.9	< 0.01
Contraction Phase (msec)	Be	95	95	0	1	0	
	Mg	78	85	+7	3	2.3	< 0.05
	Ca	87	95	+8	3	2.7	< 0.05
	Sr	90	129	+39	4	9.8	< 0.001
	Ba	87	107	+20	3	6.3	< 0.001
Relaxation Phase (msec)	Be	119	120	+1	2	0.5	> 0.1
	Mg	119	116	+3	4	0.8	> 0.1
	Ca	106	146	+40	10	4.0	< 0.01
	Sr	115	444	+329	21	15.7	< 0.001
	Ba	118	205	+87	13	6.7	< 0.001
R (arb. unit)	Be	100	83	—17	5	3.4	< 0.01
	Mg	100	65	—35	6	5.8	< 0.001
	Ca	100	49	—51	9	5.7	< 0.001
	Sr	100	67	—33	11	3.0	< 0.02
	Ba	100	61	—39	12	3.3	= 0.01
QRS (msec)	Be	31	39	+8	2	4.0	< 0.01
	Mg	32	34	+2	1	2.0	< 0.1
	Ca	27	32	+5	2	2.5	< 0.05
	Sr	28	40	+12	4	3.0	< 0.02
	Ba	34	58	+24	4	6.0	< 0.001

DISCUSSION

The concentration of the salt in question and the duration of its action are factors of importance in the present test conditions (cf. 10). The average duration of the test series was shortest in the beryllium and magnesium experiments. Therefore the action of these cations is stronger than the figures of concentrations comparing them with other salts of the present test show. The effects of beryllium and magnesium chloride are similar. Likewise the effects of calcium and strontium were very similar. Barium chloride differed somewhat from the two latter salts. The concentrations of beryllium and barium chloride tolerated by the preparation were of the same magnitude and were considerable lower than those of the other chlorides in the present study.

Comparing some previous reports, many points of contact can be found. Garb (5) found no effects on the myogram and electrogram of cat papillary muscle by magnesium. Unghváry and Obál (23) stated that magnesium diminished the injurious effect on heart muscle caused by the increase in osmotic pressure. A widening of the QRS complex has been found in animals after the intravenous injection of magnesium (20, 24).

Calcium and strontium are very similar not only chemically but also physiologically. They increase the contraction force of heart muscle (2, 4, 5, 13, 15, 17, 19, 21, 22). Garb (6) points out that in the presence of a minimal calcium ion concentration the inotropic effects of strontium are indistinguishable from the effects of adding an equimolar amount of calcium ions. He used an artificially stimulated papillary muscle of the cat. In Kruta's (14) experiments on the isolated right guinea pig auricle, the changes in the calcium chloride concentration in Locke's solution did not have any essential effect on the beating rate at various temperatures. Gross (7) found with isolated mammalian heart a retardation in the heart rate. Rutkewitsch (19) found bradycardia after the administration of calcium and strontium salts. He interpreted this as a secondary effect through the vagal centra as a result of increased blood pressure. According to him the increased blood pressure was caused mainly by the direct action of strontium on blood vessels and only a little by increased cardiac output. In the case of calcium,

the blood pressure rose as a result of the increase in stroke volume. An increase in tone caused by calcium which may lead to a permanent rigor was observed among others by Howell (8) and Luisada & Weiss (18). Electrocardiographic changes caused by calcium have inspired numerous publications; Lenzi & Caniggia (16) have listed a large number of them. Some authors have found impairment of the intraventricular conduction to result from calcium administration (1, 3, 24). In Garb's (5) experiments on the isolated cat papillary muscle, calcium did not affect the electrogram until a calcium level at which no contractile force could be recorded.

Barium is known as a very toxic ion which, although similar to calcium and strontium chemically, is quite different pharmacologically (6). However, in certain conditions it has a cardiac stimulating effect (12). In the present experiments a certain similarity of barium to calcium and strontium can be noticed. The dosage tolerated by the preparation was much lower than the dosages of calcium and strontium. According to Kisch (11), minimum effective doses of the salts of magnesium, strontium, calcium and barium retard the initiation of excitation in the frog heart. In moderate doses, strontium, calcium and barium increase this rate. Heavy doses result in powerful retardation and cessation of the initiation of excitation.

Compared with corresponding experiments with chlorides of alkali metals (10), the following conclusions can be drawn: there is hardly any specific feature in either group of cations. In higher concentrations, the cations studied diminished the R wave. Only sodium shortened the QRS interval; lithium, magnesium and calcium did not influence it and all the others caused a prolongation. The contraction force was strengthened especially by calcium, strontium and barium; small lithium and sodium doses increased it only slightly. The beating rate was retarded by all the others with the exception of sodium and calcium, small doses of which stimulated it. The contraction period remained unaltered after the addition of beryllium, magnesium and calcium; all the other cations studied prolonged it. The relaxation was slightly prolonged by alkali metal cations, very markedly by calcium, strontium and barium. Only beryllium and magnesium did not have any effect on it. On the basis of the molar concentrations tolerated by the preparation, the cations can be divided into three groups: (1) sodium

and lithium in very high, (2) magnesium, calcium and strontium in moderate and (3) potassium, rubidium, cesium, beryllium and barium only in low concentrations. This distribution does not follow the physiological importance or valence of the respective cations.

The mechanism by which the various cations of the alkaline earth group act on the mechanical and electrical phenomena cannot be explained in detail within the scope of the present study. Although certain parallellisms may be present, the various mechanical and electrical properties changed independently of each other and were influenced differently by the various chlorides. The specific action of the cations in question is apparently the most important factor in the changes observed. The increase in osmotic pressure was in all cases less than the preparation tolerates when the increase is caused by sodium chloride or glucose (9). There is hardly any common feature in the effects of the alkaline earth cations in the present study which can be explained on the basis of bivalence.

SUMMARY

The effect of alkaline earth chlorides on the mechanogram and initial deflection of the electrogram of the isolated rabbit auricle beating spontaneously in Locke's solution was studied. It was found that:

Calcium, strontium and barium chloride increased the contraction force. After beryllium and magnesium administration, only a negative inotropic effect was found.

The beating rate was retarded by all the salts studied with the exception of calcium, which gave somewhat divergent results in a moderate dosage.

The duration of isometrical contraction was unaffected by beryllium and magnesium chloride, calcium chloride prolonged the relaxation phase, and strontium and barium chloride both the contraction and relaxation phase.

The amplitude of the R wave was diminished in all the test series. Low calcium chloride concentrations gave somewhat divergent results.

The QRS interval was prolonged by beryllium, strontium and barium chloride. Magnesium and calcium chloride had hardly any effect on it.

The most important factor in the effects of the various salts studied is the specific effect of the respective cations.

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FROM THE STATE SERUM INSTITUTE, HELSINKI

STUDIES OF THE DEMONSTRATION OF INFLUENZA VIRUSES BY THEIR CAPACITY TO INACTIVATE INHIBITORS OF VIRUS HAEMAGGLUTINATION

BY

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HELSINKI 1956

HELSINKI 1956
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PREFACE

The subject of this investigation was suggested by Mr. Kari Penttinen, M.D., head of the virological department of the State Serum Institute, Helsinki. It forms a continuation of studies made earlier by him, in which I had the privilege of collaborating. I am deeply grateful to Dr. Penttinen not only for the opportunity I have had of studying virology under his keen instruction but also for his interested guidance and helpful advice in the course of the present work.

I express my sincere thanks to Professor Eero Uroma, M.D., head of the State Serum Institute, for allowing me to carry out this investigation in his Institute and for placing its facilities at my disposal. My appreciation is due to him also for the basic instruction in serology and bacteriology obtained in the scientific atmosphere of the Institute under his supervision and for his interest, valuable suggestions and constructive criticisms concerning this work.

Assistance relating to the chemical aspects of the investigation was given to me by Mr. Antti Louhivuori, Mag. Phil., head of the biochemical section of the State Serum Institute, and Mr. Esko Kaila, Ph.D., carried out the mathematical analysis of the results. I extend to both my sincere thanks for their assistance.

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Helsinki, December 1955.

Veikko Tommila.

CONTENTS

INTRODUCTION

Ever since it was first observed that the influenza virus possesses an activity resembling that of enzymes, numerous investigations have been carried out on the subject. The concept of an influenza virus enzyme has now been widely accepted in the literature. The studies of the enzymatic activity of the influenza viruses have been concerned with the interaction between the virus and the cell in order to increase our knowledge of the mechanism of infection by the influenza virus.

At first the subject of research was the nature of changes produced by the virus in the red cells and the epithelial cells of the respiratory tract. It was observed, however, that a number of biological materials contain substances which inhibit the haemagglutination by the heated virus and at the same time act as substrate of the viral enzyme. These soluble inhibitors which are distinct from the cells and which also have been isolated in the pure form, have for their part facilitated investigation work and at the same time opened up new possibilities for research.

In studying in this laboratory the capacity of small amounts of influenza virus to inactivate egg white inhibitor, it was observed that when the virus-inhibitor reaction occurred in broth or human albumin-saline solution it was possible to produce a demonstrable inactivation of inhibitor with considerably smaller amounts of virus than when the reaction took place in saline solution alone. This led to an attempt to study what are the smallest amounts of virus that can be demonstrated by means of the enzymatic activity of the virus and to investigate whether this can provide a means for a rapid demonstration *in vitro* of the influenza virus from the throat washings of a patient.

Attempts have been made in the past few years to provide a more rapid method of diagnosis of influenza by means of various

reactions, since the egg culture method which at present is most widely in use requires a number of days for the reaction. Experiments for the development of such a method have been based on the haemagglutination produced by virus concentrated from the patient's throat washings by means of red cell adsorption and elution, on the preparation of a complement fixation antigen obtained from the chick embryo infected with the patient's throat washings, or on the measurement of a reduction of the inhibition titre of the patient's nasal mucin. Quite recently, tissue culture has been used successfully for the isolation of the influenza viruses. None of these experiments have so far been applied in practice to any greater extent.

Since no attempts have previously been made to utilise for diagnostic purposes the enzymatic activity of the viruses in the patient's throat washings, and since even the least facility for shortening the time required for the diagnosis of influenza may prove helpful in combating the disease, it was considered desirable to undertake investigations with the object of studying the possibilities to use for this purpose the enzymatic activity of the influenza viruses.

REVIEW OF THE LITERATURE

INVESTIGATIONS ON THE INFLUENZA VIRUS ENZYME

THE INFLUENZA VIRUS ENZYME

Soon after the observation was made that influenza viruses cause agglutination of red blood cells (69, 93), Hirst (70) demonstrated that the viruses were adsorbed during the agglutination process onto the surface of the red cells but could be released once more under certain conditions. The adsorption occurred rapidly at a temperature of + 4°C and hardly any viruses were released at this temperature. At + 37°C, however, release was already very rapid. The treated cells were so changed that they no longer were agglutinable by the same virus. The eluated virus, however, could be re-adsorbed to an intact red cell, cause agglutination, and once more be eluated from the cells. The infective agent of the virus was adsorbed together with the haemagglutinin. Influenza viruses adsorbed onto the respiratory cells of excised ferret lung were also rapidly released (71).

On the basis of his observations Hirst suggested (71) that the release of the viruses may be the result of an enzymatic action which is necessary for the influenza viruses during their parasitic life in the living cells. The theory of the enzymatic power of the influenza viruses was thus presented for the first time. This opinion has been supported by the extensive investigations on this subject by Burnet and his co-workers and the concept of an influenza virus enzyme has now been widely accepted in the literature. This term is also used in the present report. It also has been stated that the enzymatic activity of the influenza viruses is open to question and that other explanations may be found for these phenomena (9, 64).

In addition to the above mentioned cellular »receptors», biological material of various origins was later found to act as substrate for the influenza virus enzyme (pages 13—18). Since such substrates were found to be of mucoprotein nature (96), the enzyme was at first believed to be a mucinase. Gottschalk (57, 58, 59, 60) has more recently studied in detail the reaction of the influenza virus enzyme and arrived at the conclusion that the enzymatic action is directed upon an amide link in the prosthetic group of the substrate and that the enzyme thus most nearly resembles an amidase.

Various influenza viruses have been found to differ in their behaviour towards the receptors present in the red cells as well as towards substrates distinct from the cells, or inhibitors (16, 24, 74, 118). Strain Lee of influenza B virus has proved very active; this is also true of several A strains. The mumps virus and the Newcastle disease (ND) virus have also been found to be enzymatically active, although their activity, as well as that of influenza C virus is more limited (16, 74, 75).

Already at an early stage of the study of the influenza virus enzyme it was suggested that the enzymatic activity like haemagglutinative activity, is a function of the virus particle itself (4, 52, 74). The enzymatic activity of a virus preparation purified in various manners always follows the actual virus particles, and since such activity has not been found in the host cells of the virus it has been concluded that the enzyme is an integral part of the virus particles and is not absorbed from, for instance, the host cell (54).

The enzymatic activity of strains of influenza B virus and of most strains of A virus is lost on heating to a temperature of $+ 52° — + 56°C$ for thirty minutes (48, 73, 117). After this treatment the capacity of the viruses to agglutinate red cells is still retained, but it is inhibited by inhibitors, *i.e.*, mucoproteins obtained from various biological sources. Viruses thus heated may be employed for the quantitative determination of inhibitors, and they therefore are called »indicator viruses». Various influenza viruses may be converted to indicator viruses not only by heating but also by, *e.g.*, trypsin (116) and periodate (46); certain strains of A virus require heating in citrate solution and a high pH level before they become converted to indicator viruses (36, 117). The inactivation of the viral enzyme is directly proportionate to the

conversion of the virus to indicator (16, 117), although exceptions also have been observed (112). The enzymatic activity may also be destroyed by ultraviolet rays (67).

For its action the influenza virus enzyme requires a given concentration of ions (33). Among these the calcium ion especially has been found very effective (16, 21, 35, 117), although a diverging observation has also been reported (107). Burnet (16) found that the ability of the Lee strain to inactivate purified inhibitor from ovarian cyst mucoid was much greater at + 36°C than at 0°C. He also observed that the reaction occurred within certain pH limits (pH 5.7– 7.0) and that the activity of the viruses diminished sharply above and below this range. The optimal effect was seen at pH 6.2. The rapidity of the reaction produced by the viral enzyme was directly proportionate to the viral concentration and the destruction of the substrate occurred logarithmically as a function of time. In the case of less active viruses, on the other hand, the reaction gradually became slower, so that only a portion of the substrate was inactivated. However, on the addition of more substrate the virus was again able to inactivate it. Thus the deceleration of the reaction could not be a result of the inactivation of the virus or of the development of inhibitory reaction products.

No difference has been observed in the enzyme activity of the O and D phases (20) of certain influenza A viruses against ovarian cyst mucoid (16). Virus A/BEL in the O phase was active against human ovarian cyst mucoid only, but not against ovomucin or lung suspension of the chick embryo. The D phase virus, on the other hand, was active against all three substrates. Similarly, the heated O phase virus was inhibited only by human mucoid, whereas the D phase virus was inhibited by both human and avian mucoid (119). The O phase virus has also been found to be highly active against nasal mucus inhibitor (43).

In addition to the enzyme in the influenza-mumps-ND group, a soluble enzymatic factor has been found in filtrates of certain bacteria, such as *V. cholerae* and *Cl. welchii* (1, 13, 24, 25, 95). The action of the viral enzyme and *V. cholerae* enzyme resemble each other. *V. cholerae* filtrate can destroy the virus receptors of red cells and of cells sensitive to infection and inactivate various mucoprotein inhibitors (11, 16, 24, 38, 39, 123). This agent in the filtrate

is called the receptor-destroying enzyme = RDE. RDE treatment, like virus treatment, decreases the electrophoretic mobility of cells and inhibitors (2, 65, 120). It further has been demonstrated that RDE releases from the inhibitor the same component as the virus liberates (60).

RDE is also capable of destroying influenza virus receptors from the allantois of the chick embryo and from epithelial cells of the respiratory tract of the mouse and of thus preventing their infection (114, 115). Purified enzyme of *V. cholerae* has recently been recommended for the destruction of non-specific inhibitors in serum which disturb antibody determinations (4, 80, 81, 100).

Certain proteases have also been found to possess an effect resembling that of the viral enzyme. Trypsin is capable of destroying the receptors in chicken red cells (72). Trypsin, chymotrypsin, pepsin and papain, but not ribonuclease and hyaluronidase, inactivate the serum inhibitor (96) and trypsin destroys both the so-called Francis (48) and Chu (27) inhibitors in the serum of several species of animals (110) and the human urine inhibitor (128). On the other hand, pepsin and ficin, which latter has an action similar to that of papain, have shown but little activity against the normal allantoic fluid inhibitor (126). Likewise, ribonuclease, desoxyribonuclease and hyaluronidases of various origins have no effect on it, whereas trypsin does (66).

Recent observations show that the human saliva contains a receptor-destroying factor which resembles RDE in its properties. It has been found to occur in greater amounts in the saliva of patients with poliomyelitis than of healthy persons (77, 82).

We have no knowledge of the significance of the enzymatic activity of influenza viruses. It has been suggested that following the adsorption phase and only after being enzymatically released the virus is ready for the following phase in the infection (7). However, it has been observed that the infection occurs without any outwardly visible enzymatic activity of the virus against the receptors (46).

SUBSTRATES OF THE INFLUENZA VIRUS ENZYME

Red Cell Receptors

As was mentioned above, the first observation made regarding the influenza virus enzyme concerned its action against red cells. Thus the virus receptors present at the surface of red cells were the first subject of study.

Factors possessing an inhibitory activity have been isolated from the red cells of man and some species of animals. It has been held that these factors correspond to cell receptors (10, 12, 51 72). They have not been isolated from cells treated with active viruses or cells of animal species in which haemagglutination is not produced by viruses (51, 131). The substance first isolated was determined to be chemically a lipid (10). Several factors, however, have contained a large amount of polysaccharides as well as nitrogen (12, 97, 131). The receptor substance of chicken red cells was found to be stable at a high temperature if the pH was below 10 but its activity was destroyed by sodium periodate, trypsin and influenza viruses. It was chemically of the nature of a mucoprotein and considered to be analogous to or identical with the serum inhibitor (72). McCrea (97) demonstrated that the receptors of human red cells contain hexosamine, galactose, fucose and amino acids as well as lipids.

It was observed by Hanig (65) that the electrophoretic mobility of red cells is reduced by virus treatment. This observation was extended by Ada and Stone (2, 120), who treated red cells with mumps, ND and influenza A and B viruses and with RDE. The change in mobility was characteristic of each virus and greatly resembled the receptor gradient (page 19). They suggested also a grouping of the cellular receptors according to the effect of the virus. The greatest decrease in the electrophoretic mobility was produced by RDE.

Electrophoretic changes in the red cells have been demonstrated also *in vivo* by intravenous injection of large doses of RDE into guinea pigs. Twenty-four hours after injection the electrophoretic mobility of the cells was reduced from 1.10 to 0.20 μ/sec./V./cm, and the cells were no longer agglutinated by the viruses. Their normal condition was regained in twenty days. The action of RDE

on the red cells could not be very destructive. This was regarded as evidence that at least some parts of mammalian red cells have a rapid metabolism (49).

Virus and RDE treatment alter the properties of red cells also in the respect that the treated cells are agglutinable by normal serum (3, 24). The same observation was made already earlier regarding cells treated with various bacterial filtrates (130). The factor in normal or immune serums which agglutinates treated red cells is called »T agglutinin» (19).

The action of different influenza viruses against red cells will be dealt with later in this report (page 19).

Receptors in Respiratory Tract Cells and Allantois Cells

It was demonstrated by Hirst (71) that reactions between influenza viruses and respiratory cells of the excised lung of the ferret and mouse resembled the reactions between viruses and red cells. It seemed apparent that both possessed similar receptors for viruses, by means of which the adsorption of the viruses took place but which the latter were capable of destroying enzymatically. The cells of the chorio-allantois of the chick embryo have also been found to possess virus receptors onto which the viruses are adsorbed but which may be destroyed by RDE, thus preventing infection of the embryo (114). Similarly it has been possible to prevent infection in the mouse by treating its respiratory tract with RDE before infection (115).

The action of the RDE in the respiratory tract has also been found to be based on its capacity to destroy the receptors from the respiratory cells, which then no longer are able to adsorb the viruses (38). A progressive destruction of the receptors was produced by the intranasal injection of RDE into the mouse. The receptors regained a normal condition in six days (39). In the acute stage of infection influenza viruses caused transitory destruction of the receptors in the respiratory tract of the mouse in a similar manner as RDE treatment (41).

Periodate so modified the red cell receptors and the cell receptors in the mouse respiratory tract and in allantoic membrane both *in vitro* and *in vivo* that the viruses were adsorbed but could not be eluated, but the latter two cells were infected through

the modified receptors (40, 46). The enzymatic activity of the seed virus on cell receptors and its elution from the latter has not been observed *in vivo* (37, 46, 91). From the observations made it has generally been concluded that in the first phase of infection, viruses are adsorbed onto the cells by means of the receptors, but, as already stated above, there is no definite knowledge of the part played by enzymic action in the infection (7, 46, 60, 71).

Serum Inhibitor

In addition to cellular receptors, a number of biological fluids have also been found to act as substrates for influenza viruses and as inhibitors of haemagglutination by heated viruses. Francis (48) observed that if Lee virus heated to 56°C for 30 minutes is used as the antigen in the haemagglutination inhibition test, non-specific inhibition occurred up to high serum dilutions. The factor responsible for this non-specific inhibition, the so-called »Francis inhibitor», is present in varying amounts in the normal serums of different species of animals. Chemically it is a mucoprotein, which is thermostable at a temperature of 100°C. It is resistant to most oxidative agents but inactivated by proteases and potassium periodate, as well as in the presence of RDE and influenza viruses (4, 24, 72, 96). There had already préviously been described an inhibitor in normal ferret and rabbit serum which inhibits the haemagglutination by unheated influenza A virus. This activity accompanies the globulin of normal serum and is thermolabile, being inactivated when held at + 62°C for thirty minutes (22, 94). Inhibitory components of other kinds have also been found in normal serum (80). An electrophoretically pure mucoprotein, which proved to be a highly active inhibitor, has been separated from human plasma (121). Several investigators have stressed the importance of removing non-specific inhibitors from serums used in serological tests (68, 80, 81, 100).

The Egg White Inhibitor

Substrates of as high purity as possible are indispensable for the study of the reaction between the viral enzyme and the substrate. A variety of methods and a number of inhibitors from

different sources have been employed for their production. The suitability of the egg white inhibitor for this purpose has been widely investigated.

Gottschalk and Lind (61) demonstrated that ovomucin is the only component of egg white which has an inhibitory action on the influenza virus. Its isolation has been described by Young (133). Ovomucin contained 15 per cent of lysozyme, which could be removed by crystallisation. The influenza viruses, *V. cholerae* enzyme and trypsin were capable of inactivating the ovomucin inhibitor. The inhibitory component comprised only 5—10 per cent of the total ovomucin fraction (45).

Lanni *et al.* (86, 87, 88, 90) studied in several investigations the characteristics of egg white inhibitor. Using phosphate precipitation they prepared this inhibitor, which had a purification factor (PF) of 62 based on nitrogen. The inhibitory component, which had a PF value of 49, was separated from the egg white by ultracentrifugation. According to the investigations of these workers, the inhibitory activity was closely associated with a viscous, thermostable, non-dialysable component, which was of the nature of a carbohydrate complex. Its analysis gave the following values: Nitrogen, 12.5 per cent; sulphur, 2.4 per cent; carbohydrates, 10.3 per cent; no phosphorus. Two components were distinguished electrophoretically. On ultracentrifugation at 67,000 r.p.m. for 90 minutes 80 per cent of the active component was sedimented to the bottom. The sedimentation constant was 31—37S. Under the electron microscope the inhibitor particles were 10—20 mμ in size, and their ellipsoid axial ratio was 1: 90 and molecular weight 7.6×10^6. It was calculated that three of these particles were capable of neutralising one virus. From semi-purified egg white inhibitor Sharp *et al.* (111) separated electrophoretically three components, two of which were active. The specific activity of the most active component was 190.

In addition to the above, a filtration method for purification of the egg white inhibitor has also been described, enabling the preparation by a simple procedure of large amounts of inhibitor with a high PF (29). In addition to filtration, deproteinisation with chloroform and precipitation with ethanol have also been used. The active product thus obtained contained 26 per cent of carbohydrates and 73 per cent of proteins. Under the electron micro-

scope the particles were seen as filaments. The active virus destroyed the activity of the product at + 37°C (85). Inhibitors with a PF varying between 124 and 432 on the basis of nitrogen were prepared by the ultracentrifugation of a semi-purified inhibitor obtained from egg white by precipitation with distilled water at + 4°C (103).

Urine Inhibitor

Tamm and Horsfall (128, 129) isolated in various manners an inhibitor from normal human urine and studied its biological, chemical and physicochemical properties and the reactions between this inhibitor and influenza viruses. The inhibitor concentration in the urine could be 2 mg per cent. It was still active at a concentration of 0.0001 μg per cc. Viruses of the influenza-mumps-ND group destroyed its activity. The inhibitor, which was found to be a mucoprotein of high molecular weight, was homogeneous in electrophoresis and when treated with an active virus its electrophoretic mobility declined by *c.* 20 per cent (106). The reactions between the urine inhibitor and viruses and between this inhibitor and RDE resembled those of other inhibitors. However, this inhibitor differed from other inhibitors in its poor water-solubility and its low capacity to agglutinate normal as well as RDE-treated red cells (18, 106).

It was believed that even the smallest carbohydrate unit in the urine mucoprotein contains 6 hexosamines, 4 galactoses, 2 mannoses, 1 or more fucose residues, and possibly also an additional component, and that the prosthetic group is the smallest unit (2,500) *n* in which *n* could be no greater than 3 or 4, rather than a giant polysaccharide. The urine mucoprotein possibly has 200 of these small carbohydrate complexes at the surface of one protein molecule (57). The viscosimetric behaviour of the urine mucoprotein has also been described (30).

Normal Allantoic Fluid Inhibitor

The nature of the normal allantoic fluid inhibitor was studied by Svedmyr (122—126) in a number of investigations and by Hardy and Horsfall (66). The normal allantoic fluid of a 7-day

or older chick embryo was found capable of inhibiting haemagglutination by influenza viruses. Infection of the embryo with the influenza virus gradually diminished the inhibitory activity of the allantoic fluid. The inhibitive activity was connected with a component in the allantoic fluid which had a sedimentation constant of about 200S (122) and which remained stable at high temperatures and over a wide pH range (66). Both the active and the inactivated purified influenza virus combined with the purified inhibitor, forming a precipitate. Of the two combinations, that of the inactivated virus remained stable. The presence of antibody prevented combination (124, 125). Treatment of the inhibitor *in vitro* with small amounts of active virus, sodium periodate or filtrate of *Cl. welchii* gradually destroyed both the inhibitory power and the capacity to be precipitated with the virus (123, 126). Svedmyr postulated that one virus particle was inhibited by one allantois inhibitor particle (125). Even large amounts of inhibitor added to the inoculum did not prevent the infection of the chick embryo (123). The effect of enzymes on the allantois fluid inhibitor was dealt with earlier (page 12).

Inhibitors from Various Sources

A variety of mucinous materials, such as ovarian cyst mucoid and the secretion of the sheep salivary gland have been found to contain a large amount of inhibitor (5, 14, 15, 16, 23, 50). The inactivation of the inhibitory capacity of ovarian cyst mucoid when treated with active influenza virus has been studied from several aspects (16).

Two inhibitors of haemagglutination and one factor that neutralised the infection in the egg have been demonstrated in sputum (92). The human nasal mucus has a fairly high content of inhibitory substance against various indicator viruses; this substance is destroyed enzymatically by active viruses (43). An inhibitor with a high titre has been isolated from the sputum of patients with chronic bronchitis (32).

Curtain *et al.* (31) prepared from meconium a highly active water-soluble inhibitor. When lyophilised it formed a white powder with an activity of 50,000 inhibition units per mg dry weight. Various influenza viruses and RDE destroyed the activity of pure

inhibitor. As the meconium inhibitor is soluble in water it has been found particularly suitable for use in viral enzyme investigations, especially in studying the importance of ions and ion concentrations.

On the subject of substrates of the influenza virus enzyme mention should also be made of the observation that the active influenza virus was capable of inactivating gonadotropic hormones in the serum (132). This finding was evidence supporting the opinion that the substrates are of mucoprotein nature. Carbohydrates of various origins, such as apple pectin, citrus pectin, flaxseed mucilage, blood group A substance, gum acacia and gum myrrh, as well as red cell extract inhibited haemagglutination by influenza A virus (63). Several other polysaccharides of animal and vegetable origin were also found to possess the same capacity (79). The enzymatic activity of the virus against the last mentioned substances has not been studied.

Receptor and Inhibitor Gradients

Burnet *et al.* (24) observed that the treatment of red cells with influenza viruses or RDE may change the cells so that they no longer are agglutinated by certain influenza viruses, whereas others still agglutinate them. Each virus destroys only a certain proportion of the cellular receptors. The viruses may accordingly be arranged in a linear series on the basis that a cell rendered insusceptible by the action of any given virus is not agglutinated by viruses which precede this virus in the series, but is agglutinated by all those which follow it. They designated this as the receptor gradient. The order of the series was as follows: Mumps virus, ND virus, influenza A viruses, influenza B viruses, and swine influenza virus. The gradient obtained by RDE treatment differed chiefly in respect to the mumps virus from that obtained by virus treatment. The above mentioned workers were the first to draw attention to the possible complexity of virus receptors in the red cells and to the possible presence of different enzymes in the different viruses.

A corresponding inhibitor gradient was presented by Stone (118) for soluble inhibitors. By treating inhibitors from various sources with different viruses he determined the residual inhibitor with

the aid of three different indicator viruses. When treated with different viruses the inhibitor lost its activity against different indicator viruses in the same given order, which nevertheless varied for the different inhibitors. A similar gradient as with virus treatment was obtained for ovomucin with RDE treatment. Burnet (17) found in soluble inhibitors a specific relationship between the active virus and the corresponding indicator virus, so that each active virus reduces the activity of the inhibitor against the homologous indicator virus to an extent beyond that which corresponds to its place in the gradient.

The gradient problem in particular was studied by Hirst (74), who observed that each virus examined in the mumps-ND-influenza group (5 strains) had the possibility of destroying the receptors for all viruses from human blood plasma and egg white inhibitor if adequately large amounts of virus were allowed to act for a sufficient length of time. Mumps and ND viruses, however, had a more limited destructive activity against receptors. The rate of destruction differed with the different viruses, but the order of destruction was similar. Hirst concluded that different viral enzymes are very similar but that there are a number of kinds of receptors; however, he expressed doubts concerning the existence of a fundamental over-all gradient series. The influencing factors of the inhibitor gradient have been pointed out by Lanni *et al.* (89). It has been observed that the receptor gradient changes according to the length of time of virus treatment (47).

Capacity of Substrates to Prevent Infection

When the virus was diluted with the inhibitor solution, 300 μg of urine mucoprotein per embryo was able to reduce the infection titre (Lee) from $10^{-7.5}$ to $10^{-5.5}$. When 200 μg of the same inhibitor was injected two hours before injection of the virus the infection titre was reduced by 1—2 logarithms and the haemagglutination titres were 4—16 times lower than usually (129). The purified ovarian cyst mucoid reduced markedly the infectivity of influenza A virus in the mouse and the chick embryo, and treatment with periodate increased the capacity of cyst mucoid to inhibit infection in the chick embryo (15). Apple pectin inhibited the multiplication of influenza A virus in the egg (63). Human sputum has also been

found to have an inhibitory factor capable of preventing infection by the influenza virus (8, 108). Even large amounts of allantois inhibitor were unable to inhibit the infection of the chick embryo (123).

REACTION BETWEEN THE INFLUENZA VIRUS ENZYME AND THE SUBSTRATE

The basic observation made regarding the action of the influenza virus enzyme was the finding that influenza viruses adsorbed onto the surface of red cells are able to become released from the cells. At the same time the cells undergo change, so that they can no longer be agglutinated by the same virus. Receptor substance cannot be extracted from red cells treated with virus, their electrophoretic mobility is reduced, and the treated cells are agglutinated by normal serum. Heated virus is adsorbed by red cells but cannot be eluated from them. Similarly, the viruses are not eluated from cells treated with periodate. The reactions of the receptors in the allantois and respiratory cells resemble those of red cell receptors. In the same manner as the receptors are destroyed from red cells by an active virus and RDE, they are destroyed by RDE also from the epithelial cells of the mouse and ferret respiratory tract and the allantois cells of the chick embryo, which subsequently do not become infected.

The discovery and purification of the soluble inhibitors has made a transition possible from biological to chemical methods in the study of the reaction occurring between the viral enzyme and the substrate. This subject was studied especially by Gottschalk in several investigations.

In similarity to cell receptors, the mucoprotein inhibitor is inactivated and its electrophoretic mobility falls slightly when the active virus acts under given conditions (106, 129).

Gottschalk and Lind (62) demonstrated that the influenza A virus liberated from ovomucin a dialysable component containing carbohydrate and nitrogen, which was identified at first as a N-substituted hexosamine. The Lee virus similarly liberated from electrophoretically homogeneous urine mucoprotein a component that resembled that obtained from ovomucin (55). N-substituted glucosamines were found to be commonly present in the mucopoly-

saccharide or mucoprotein components produced by cells, fungi and bacteria (56) and a similar component has been reported to have been liberated from ovomucin simply by mild alkali hydrolysis (113).

A positive Ehrlich's reaction was obtained with the nitrogen-containing portion of the component separated by the virus from the inhibitor and this component has now been identified by Gottschalk (58, 59, 60) as 2-carboxy-pyrrole, which forms an amide link with the hexosamine residue and a glycosidic bond with the sugar residue. He found that all the mucoproteins examined by him — whether inhibitors or not — contained this group. The composition of the carbohydrate complex of the urine mucoprotein is thus presumed to be as follows: 8 hexosamines, 6 galactoses, 3 mannoses, 1 fucose, 4 2-carboxypyrrole residues. The molecular weight unit would be $(3264)n$, where n is <5. There may be 200 small prosthetic groups of this kind on the surface of each protein molecule.

By action of the mutual electrostatic forces prevailing between the enzyme groups on the surface of the viral particles and the prosthetic groups of inhibitors or receptors, the viruses are adsorbed onto the inhibitors or cells. The adsorption is followed by the enzymatic action, which is directed against the above mentioned amide linkage in the prosthetic group (60).

TESTS FOR THE RAPID DIAGNOSIS OF INFLUENZA

Egg culture has become the most widely used method for isolation of the influenza virus from throat washings. However, several days or even a week are required before a sufficient amount of haemagglutinin for demonstration and identification of the virus under »standard» haemagglutination conditions is produced in the egg.

It is thus comprehensible that efforts have been made to develop more rapid methods for demonstration of the virus. Kalter (83) reported that with red cell adsorption and elution for concentration of the virus and with specific antiserum he had been able in several cases to identify the influenza virus from the patient's throat washings. He considered this a rapid method for the diagnosis of

influenza during epidemics. However, it has not been possible to confirm the serviceability of the test (26, 53).

Hummeler *et al.* (78) prepared from the allantoic fluid or membranes of a chick embryo infected with the patient's throat washings an antigen which they employed in the complement fixation test with known antiserums. By this procedure they were able to identify the virus 72 hours after the sample was taken.

Fazekas de St. Groth (42, 43, 44) studied the possibility of employing for diagnostic purposes the changes occurring in the inhibition titre of the nasal mucus of influenza patients. Virus in amounts which were considerably smaller than the agglutinating dose were capable of destroying the nasal mucus inhibitor *in vitro.* On the basis of this finding he tested the inhibition titre of the nasal mucus of influenza patients and found it to be lower than that of healthy subjects. The changes in the inhibitory capacity of the nasal mucus of influenza patients had a positive correlation to the objective findings in influenza virus infection. In 80 per cent of the cases the amount of inhibitor was markedly reduced in the acute phase of the disease but reverted to normal during the convalescent stage. Certain other respiratory diseases studied by him did not produce similar changes in the nasal mucus inhibitor.

The sensitivity of this test was nearly as high as that of other tests for influenza (egg culture, haemagglutination inhibition test, and complement fixation test). The investigator therefore considered the test to be an objective one in influenza virus infection. However, he regarded it as a presumptive rather than a definitive test.

Recently, tissue culture has given promising results in the isolation of influenza viruses from the throat washings of patients when human embryonic kidney or lung tissue (98, 99) or monkey kidney (127) is used. Identification of the virus has been possible by this method in three to seven days. It has proved serviceable especially for the isolation of influenza B viruses, for which a positive reaction was obtained in 21.8 per cent, whereas egg culture gave a positive reaction in 1.3 per cent only. In the isolation of influenza A virus, however, egg culture was found to be better than tissue culture. The influenza C virus has also been isolated by tissue culture in a number of cases.

CONCLUSIONS FROM THE LITERATURE

It is widely acknowledged that the influenza viruses contain an enzyme which is not separable from the viral particles. Substrates of the enzyme have been found in red cells, in cells sensitive to influenza virus infection, and in many biological fluids. These inhibitor substrates have been found to be chemically of mucoprotein nature. They are highly stable to heat and changes in pH, but their activity is easily destroyed by periodate, several proteolytic enzymes, some bacterial enzymes, and influenza, mumps and ND viruses. Inhibitors of various origins differ from each other in respect to certain biological and physical properties. The prosthetic group of the inhibitor-substrates is a complex carbohydrate, which in addition to simple and amino sugars contains a 2-carboxy-pyrrole group, which is common for several of the inhibitors examined. The action of the influenza virus enzyme is believed to be directed against the amide link between the 2-carboxy-pyrrole group and hexosamine.

In infection with the influenza virus the first phase is the adsorption of the virus onto the surface of the cell by means of the enzyme groups of the virus particles and the prosthetic groups of the receptors. If the adsorbed virus is active, the adsorption will be followed by release of the virus by the action of the enzyme and the destruction of the receptors. If the cell receptors are modified by periodate so that the viruses, after adsorption will be incapable of elution or are eluated very slowly, the cell will nevertheless become infected. It would thus appear that the action of the viral enzyme against that portion of the receptors by means of which the viruses are adsorbed is not necessary for infection of the cell. In live experimental animals, also, the adsorption of the viruses onto cells sensitive to infection is not followed by liberation of the viruses. Although the enzymatic action of influenza viruses on the cell receptors and inhibitors has already been clarified in detail, the ultimate significance of the enzyme in influenza virus infection still remains obscure.

In the research work on the enzymatic reaction of the influenza virus the practical application of the reaction has been given less study. There still remains open, among other points, the question

of the smallest amount of virus that under suitable conditions is capable of inactivating a given amount of substrate. If sufficiently small amounts of virus can be demonstrated, the reaction may find application for instance as a diagnostic aid. Rapid tests so far developed for the diagnosis of influenza are a fairly recent development and their value in clinical practice has not yet been proved.

PROBLEMS OF THE PRESENT INVESTIGATION

The object of the present investigation was to endeavour to obtain information on the following problems:

1) Determination of the capacity of influenza virus concentrations below the haemagglutinative titre to inactivate the viral haemagglutination inhibitor under variable conditions, as a means for the demonstration of minimal amounts of virus;

2) Determination, based on the foregoing reaction, of the capacity of throat washings to inactivate the viral haemagglutination inhibitor, with a view to using this capacity as a diagnostic test for influenza.

PRESENT INVESTIGATION

STUDIES OF INACTIVATION OF VIRUS HAEMAGGLUTINATION INHIBITOR BY LOW CONCENTRATIONS OF INFLUENZA VIRUS

Relatively large amounts of reacting components have so far been used in studies of influenza virus enzymes, in order to facilitate the determination of the changes which occur, to obtain accurate results and to employ a short reaction time. The influenza virus concentrations used have been higher than the agglutinating concentrations. However, a demonstrable inactivation of inhibitor has also been observed with lower concentrations of the virus (43).

The capacity of small amounts of influenza virus to inactivate the egg white inhibitor has been found in this laboratory to be greater in broth and in albumin-saline solution than in saline solution (104). By carrying out the virus-inhibitor reaction in these solutions it was possible to cause a demonstrable inactivation of the inhibitor by virus dilutions which were considerably below the haemagglutinating concentration. However, it was necessary to use a long reaction time of 24 hours. Long reaction times can be employed provided infection is prevented by the use of antibiotics.

Since in this manner it was possible to demonstrate virus amounts so small that the application of the method to diagnostic tests appeared practicable, it seemed warranted to conduct a closer study of the capacity of influenza virus concentrations below the agglutinating titre to inactivate the inhibitor under variable conditions, and to obtain information on the smallest amounts of virus demonstrable under optimal conditions by means of their enzymatic property.

MATERIAL AND METHODS

This study was made chiefly with influenza virus A/Finland/1/51, since most of the influenza epidemics have been ascribed to A viruses and since virus A/Finland/1/51 has been found to possess an effective enzymatic activity (104). The indicator virus was chiefly the Lee strain of influenza virus B, which has proved sensitive as an indicator (16, 74). Most of the tests were carried out in broth. Since broth contains unknown components and since the composition of different batches is variable, it naturally is not a very suitable medium for the study of enzymes. However, since it is commonly in use in bacteriological and virological laboratories and is readily procurable, and since furthermore this investigation was made with a view to practical applications, the use of broth was considered justified.

The Virus

The influenza virus strain used in these studies was A/Finland/1/51/E 25—34, designated briefly in this report as A/F/51. Since highly dilute virus concentrations, *i.e.*, infected allantoic fluid dilutions 10^{-3}—10^{-6}, were employed, the infected allantoic fluid was used as such, as it previously has been found suitable for this purpose. No marked difference has been found in the enzymatic behaviour of the virus in raw allantoic fluid and dialysed allantoic fluid (16). The virus preparation was made as follows: Ten-day-old chick embryos were infected by injection into the allantoic sac of 0.2 ml of allantoic fluid virus dilution 10^{-3} in broth, also containing 500 units of penicillin and 500 γ of streptomycin per millilitre. After three days' incubation (+36°C) the allantoic fluids were collected and pooled. The infected fluids were stored at +4°C and used as fresh as possible. The haemagglutination titres of the pooled allantoic fluids when tested with a 0.5 per cent suspension of chicken red cells ranged from 1/2048 to 1/4096. Some tests were made with Lee strain of influenza B (F 8 M 139 E 175—176), which was prepared and stored in the same manner as the A/F/51 virus.

Indicator Virus

The indicator virus was allantoic fluid of the chick embryo infected in the above described manner with the same Lee strain (E 166—176) and inactivated by heating at +56°C for 30 minutes (117). In some tests made with virus A/F/51 as indicator, the virus was found to be converted to indicator by the same treatment as the Lee virus.

Inhibitor Substrate

Egg White Inhibitor. — Using the method published earlier (103), a purified inhibitor was prepared from egg white, as follows: Into 50 ml of egg white filtered through gauze and cooled to +4°C was added 1.5 volume of distilled water +4°C under constant stirring. On the following day the white caseous precipitate was separated by centrifugation at 3,000 r.p.m. for 5 minutes and washed three times with water at +4°C. It was then dissolved in 0.06 M phosphate buffer for *c.* 24 hours, shaking occasionally. The resulting viscous fluid was centrifuged until clear at 1,500 r.p.m. for 15 minutes. The supernatant fluid was ultracentrifuged (Spinco model L) at 35,000 r.p.m. for 30 minutes (average centrifugal force = 80,730 × g). The small amount of transparent mass that formed a sediment at the bottom of the tubes was dissolved in phosphate buffer during 24 hours and shaken from time to time. When centrifuged clear at 1,500 r.p.m. for 15 minutes it yielded 20 ml of inhibitor having a titre varying between 1/320 and 1/640 when titrated against Lee indicator virus and chicken red cells.

In some experiments the substrate used was egg white diluted 1/100, filtered through gauze and centrifuged at 1,500 r.p.m. for 5 minutes.

Meconium Inhibitor. — This was prepared mainly along the lines of the method described in the literature (31). A total of 120 g of meconium collected from five newborn and 220 ml of 90 per cent phenol were homogenised in a blender. Homogeneous phenol suspension was briskly shaken in a mixing funnel with 250 ml of a saturated sodium acetate solution and then centrifuged at 2,500 r.p.m. for 2 hours. The clear, yellowish fluid obtained was dialysed at + 4°C for 48 hours against distilled water changed three times. This yielded a clear, yellowish fluid which contained *c.* 4 mg of dry substance per millilitre. When Lee indicator virus was used, the titre of the inhibitor ranged from 1/10,000 to 1/20 for different chicken red cells (105). The fluid was lyophilised at — 70°C and stored at + 4°C. For the experiments a solution containing meconium inhibitor in the amount of 0.3 mg/ml was used.

Chicken Red Cells

In tests with inhibitors, the red cells of different chickens have been found to react differently, as specifically pointed out by Penttinen *et al.* (105). The cells were obtained from chicken blood drawn by heart puncture or from the wing vein into a 3.5 per cent sodium citrate solution. In a 10 per cent concentration at + 4°C the thrice washed cells remained serviceable for one week provided handling was sterile. The tests were performed with a 0.5 per cent cell suspension. Chicken cells sensitive to meconium inhibitor were selected for the tests with this inhibitor.

Antiserums

The antiserums were chicken serums obtained by infecting chickens intravenously and intramuscularly with influenza virus A/F/51 or Lee. The haemagglutination inhibition titres were 1/320 for the A/F/51 antiserum and 1/640 for the Lee antiserum. To destroy non-specific inhibitors most of the serums were treated with *V. cholerae* filtrate (Cholera filtrate, Batch 9251, N. V. Philips-Roxane) by incubation of 5 parts of filtrate and 1 part of serum for 18 hours at + 37°C and for 1 hour at + 56°C. Some of the serums were treated with crystalline trypsin (8 mg/ml) at + 56°C for 30 minutes (110). With either treatment the inhibition titre of normal chicken serum declined below 1/20 when tested with Lee indicator virus.

Reaction Media

Phosphate Buffer. — This was 0.06 M Sørensen phosphate buffer, pH 7.2.

Saline Solution. — A 0.9 per cent NaCl solution was buffered with 0.06 M phosphate buffer (1/10th part buffer).

$CaCl_2$, $MgCl_2$ and NaCl (pro anal.). — Solutions of these substances were prepared just before each test in which they were employed.

Broth. — The broth contained 3 g of meat extract (Liebig), 10 g of peptone (Witte) and 5 g of NaCl in 1,000 ml of distilled water sterilised in an autoclave. The pH was adjusted to 7.0.

Determination of Haemagglutination Titre

On a plastic plate (109), serial two-fold dilutions of infected allantoic fluid were prepared in volumes of 0.25 ml. To each dilution there was added 0.25 ml of red cell suspension and the whole was mixed by shaking. After one hour at room temperature the results were read. The dilution which produced complete agglutination of red cells was regarded as the end point.

Titration of Inhibitory Activity

Titration of the inhibitory activity of the inhibitor substrates was carried out on a plastic plate (109). Primary dilutions of 1/20 were made with saline, from which two-fold dilution series in volumes of 0.25 ml were prepared. To each dilution were added four agglutinating units of indicator virus in 0.25 ml and the whole was left for 30 minutes at room temperature (+ 18°C), after which 0.5 ml of the red cell suspension was added. When the results were read one hour later, the last inhibitor dilution which completely inhibited haemagglutination by the virus was considered the end point. For tubes showing only *c.* 50 per cent inhibition the result was interpolated at the logarithmic mean of the dilutions. A virus control made in connection with each titration indicated that the amount of virus employed was four agglutinating units.

In the experiments with virus the inactivation of the inhibitor substrate was determined similarly by testing the inhibition titre before and after the experiment. The virus was not destroyed before titration since, with the exception of dilution 10^{-3}, the virus concentrations used in the test were lower than the agglutinating concentration, and since a further four-fold dilution occurred already at the beginning of the titration. The dilution series was started with the substrate dilution used in the test without further dilution. In tests containing antiserums, inactivation was effected by keeping the tubes at $+ 100°C$ for 2 minutes.

Test Technique

Most of the tests were carried out with virus dilutions 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Into a test tube were placed 0.5 ml of virus dilution and 0.5 ml of inhibitor dilution and shaken. The tubes were closed with a stopper and incubated in the water-bath at $+ 37°C$ for 24 hours. The titre of the residual inhibitor was then determined and the result was compared with the titre of a control tube which did not contain virus. The titre of the control tubes was not found to have declined in any of the cases.

In all the tests the reaction medium contained 500 units of penicillin and 500 γ of streptomycin per millilitre. In saline solution these antibiotics had no effect on the reaction. In broth, however, it was difficult to carry out comparative experiments without the addition of antibiotics.

Accuracy of the Method

To control the accuracy of the method the inhibitor destruction test described above was repeated eleven times. With a known concentration of inhibitor a minus result was obtained in eleven cases; the following lower concentration, which was one-half of the preceding concentration, gave a minus reading in nine cases and $\pm$ in two cases, and the next lower concentration, which again was one-half of the preceding, gave plus in eleven cases. According to these observations, a control of the accuracy of the method was possible by using as the ideal interval an interval which converted minus values to plus values.

In the present series this ideal interval was in nine cases log 2 and in two cases 2 log 2. This will give the following mean ideal interval and standard deviation of the mean (page 57):

$$1.182 \log 2 \pm 0.122 \log 2, \text{ or } 0.356 \pm 0.0367.$$

On this basis, the confidence limits corresponding to 95 per cent probability will be:

$$0.356 \pm 2.23 \; 0.0367 = 0.274 \cdots 0.438.$$

Since the interval used, $\log 2 = 0.301$, lies within the above range, it may be regarded as a suitable interval.

The graphs for the results contain also the $\pm$ readings, which should be clearly differentiated from + and — results. Such $\pm$ results are placed in the graphs half-way in the step log 2, but they cannot be considered to have any significance as accurate results.

INACTIVATION OF INHIBITOR BY LOW CONCENTRATIONS OF INFLUENZA VIRUS A/F/51 IN SALINE AND BROTH

To obtain confirmation of the earlier observation that inhibitor is destroyed in broth and in albumin-saline by lower virus concentrations than in saline, the logarithmic curves of the mean values were calculated on the basis of several different tests which were carried out in broth and saline by an identical technique under identical conditions. The substrates used were purified egg white inhibitor and diluted egg white. The results of the experiments on inactivation of meconium inhibitor by influenza virus A/F/51 in broth and saline are also presented.

Test Technique. — In these experiments, ten-fold serial dilutions were prepared of influenza virus A/F/51 in broth and saline, the final dilutions being 10^{-3}—10^{-6}. The egg white inhibitor (10 tests) was diluted in the same media to give a final titre 1/64; in some of the tests the substrate was egg white diluted 1/100 (6 tests). Then 0.5 ml of diluted virus and 0.5 ml of diluted inhibitor were combined and the test proceeded as usual (page 31). A similar test was also made with meconium inhibitor diluted to 0.3 mg/ml.

The results obtained in tests with the egg white inhibitors are shown in figs. 1 A and 1 B.

Using egg white inhibitor as substrate, inactivation of the inhibitor was produced in saline by the different dilutions of virus as follows, the mean inactivation values being stated: 10^{-6}, 0; 10^{-5}, 0.02 ± 0.025, log; 10^{-4}, 0.05 ± 0.050, log; and 10^{-3}, 0.54 ± 0.075, log. In broth the following results were obtained: 10^{-6}, 0.17 ± 0.039, log: 10^{-5}; 0.28 ± 0.027, log; 10^{-4}, 0.70 ± 0.074, log, and 10^{-3}, 1.29 ± 0.128, log. Thus a clearly demonstrable inactivation in saline was not produced by greater dilutions of the virus than 10^{-3}, whereas in broth already the dilution 10^{-5} caused a decline of nearly 0.3 (0.28), log in the inhibitor titre, which signifies that nearly 50 per cent of the inhibitor was inactivated. Even the dilution 10^{-6} caused a fall of 0.17, log in the inhibition titre. Thus it may be said that 10- to 100-fold differences exist between the inactivation of inhibitor in broth and in saline.

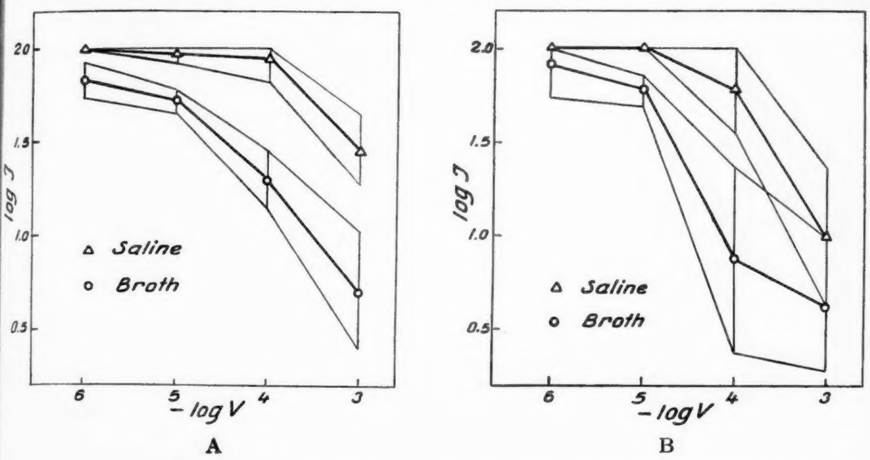


Fig. 1 A and B. — Graph showing mean inactivation of egg white inhibitor (A) (10 tests) and native egg white diluted 1/100 (B) (6 tests) by low concentrations of influenza virus A/Finland/51 in broth, pH 7.0, and saline during 24 hours at +37°C. Titrations carried out with Lee indicator virus. Confidence limits drawn with 95 per cent probability.

I = Percentage of residual inhibitor
V = Virus dilution

When diluted egg white was used as substrate, the difference between the results obtained in broth and saline was not as distinct. In the higher concentrations (10^{-4} and 10^{-3}) the virus was definitely more active in both media than in the preceding tests. The different dilutions of virus produced, on an average, inactivation of the inhibitor in saline as follows: 10^{-6}, 0; 10^{-5}, 0; 10^{-4}, 0.22 $\pm$ 0.075, log; and 10^{-3}, 1.01 $\pm$ 0.112, log. In broth the mean values were as follows: 10^{-6}, 0.09 $\pm$ 0.060, log; 10^{-5}, 0.22 $\pm$ 0.034, log; 10^{-4}, 1.13 $\pm$ 0.195, log; 10^{-3}, 1.38 $\pm$ 0.122, log. Diluted egg white, which also contains components other than inhibitor, enhanced the reaction, so that the difference in the inactivation of inhibitor in saline and broth was only about 10-fold.

The result obtained in the test with meconium inhibitor are shown in fig. 2. With this substrate no notable difference is seen in the action of the virus in experiments carried out in broth and saline. However, the inactivation of the inhibitor in broth by virus dilution 10^{-3} was of slightly higher order than in saline. The virus did not show as great activity against meconium inhibitor as against

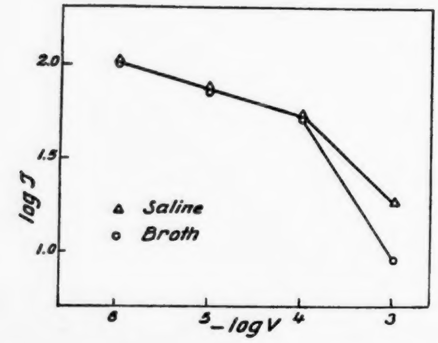


Fig. 2. — Graph showing inactivation of meconium inhibitor by low concentrations of influenza virus A/F/51 in broth, pH 7.0, and saline during 24 hours at + 37°C. Titrations carried out with Lee indicator virus.

I = Percentage of residual inhibitor
V = Virus dilution

egg white inhibitor, when Lee indicator virus was used in the titrations.

Experiments were made to determine whether the differences seen in the inactivation of the inhibitor in broth and saline were attributable to the titrations carried out in the two media. Egg white inhibitor was treated in saline with different dilutions of influenza virus A/F/51 in the same manner as in the previous experiments (page 32). Titration of the residual inhibitor was then carried out by two techniques, *i.e.*, as usually (page 30), and

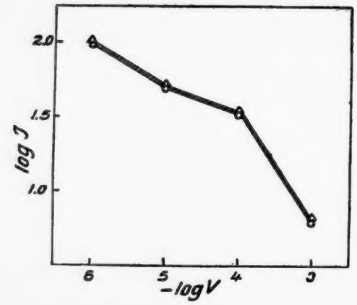


Fig. 3. — Graph showing the similar results obtained when the inactivation of egg white inhibitor treated with A/F/51 in saline during 24 hours at +37°C was titrated in both broth, pH 7.0, and saline.

I = Percentage of residual inhibitor
V = Virus dilution

otherwise in the same manner with the exception that the first dilution of the inhibitor dilution series was made into broth and the series was then continued with saline, thus corresponding to the titrations with the inhibitor diluted in broth.

The results for this control experiment are seen in fig. 3. A fully similar result was obtained in both media, which indicated that the greater inactivation produced by the virus in broth as compared to saline was not a result of the titration technique.

STABILITY OF THE ENZYMATIC ACTIVITY OF THE VIRUS DURING THE REACTION

It has been observed that the enzymatic activity of the different influenza viruses is inactivated within a relatively short time at + 52—56°C, depending upon the inactivating medium (page 10). Since the long incubation time of 24 hours was used in the present investigation, considerable inactivation of the viral enzyme was to be expected although the incubation temperature was only + 37°C. However, previous investigation did not demonstrate definite inactivation during a similar incubation time (104). This subject was studied further in the present investigation by using virus concentrations higher than the haemagglutination titre, since this made it possible to employ a short reaction time in demonstrating enzymatic activity in the virus before and after incubation at + 37°C for 24 hours. The test also demonstrated the ability of concentrations of virus higher than the agglutinating concentration to destroy inhibitor in broth and saline.

Test Technique. — Since the agglutinating unit has been employed for measurement of the virus in most studies on viral enzyme reported in the literature, the virus dilutions used in the present investigation corresponded to 0.1—1,000 agglutinating units.

Ten-fold serial dilutions of virus A/F/51 were made with broth and saline; the final dilutions were 1/8—1/80,000. The first tube in each series of the test thus contained 1,000 agglutinating units of virus and the last tube 0.1 unit. Egg white inhibitor was diluted with the same media to give a final titre 1/64 for use as substrate. The experiment was then carried out in the usual manner (page 31) but with incubation at + 37°C for only one hour, after which the titre of the residual inhibitor was determined. When the above mentioned virus dilutions had been kept in the water

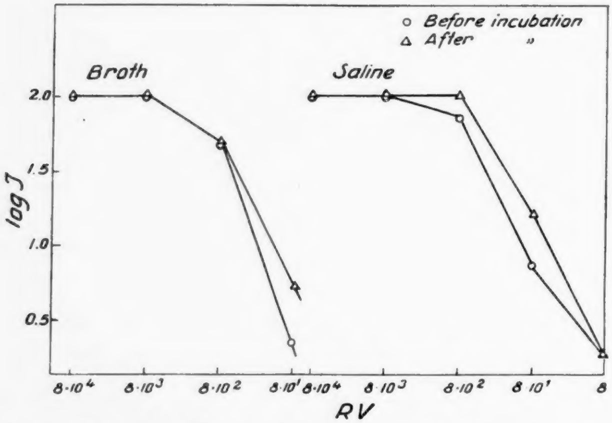


Fig. 4. — Graph showing changes during 24 hours in the enzymatic activity of influenza virus A/F/51 at +37°C in broth, pH 7.0, and saline.

I = Percentage of residual inhibitor
RV = Reciprocal of virus dilution

bath at + 37°C for 24 hours, the capacity of the virus to destroy inhibitor was determined once more in the manner described above. The readings were then compared with the controls as before.

The results are shown in fig. 4, from which it will be seen that also in this test in which the reaction time was only one hour the viral enzyme was more active in broth than in saline, even if the difference was not very marked. Ten agglutinating units (dilution 1/800) produced in broth a fall of 0.3 log in the inhibition titre and a smaller decline in saline. The difference was even more clear-cut when 100 agglutinating units (dilution 1/80) were used. Incubation of the virus at + 37°C for 24 hours reduced the enzymatic activity of the virus only slightly. There was no essential difference in this activity in the two media. However, when 100 agglutinating units were used, a definite drop of *c.* 0.3 log was seen in the inhibition titre in both media after incubation of the virus.

The result obtained is evidence in support of the earlier observation that the enzyme of the influenza virus is to a great extent resistant in saline and broth to a temperature of + 37°C.

EFFECT OF pH

The great significance of pH in an enzymatic reaction is well known. Its influence on reactions of the influenza virus enzyme

has also been studied, and the pH range 5.7—7.2 has been found suitable for the activity of this enzyme (16).

To study the effect of the pH of the broth on the reaction of the influenza virus enzyme, a series of broths with the following pH values was prepared: pH 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. An inhibitor inactivation test was carried out with each broth, using the usual technique (page 31), a virus dilution series of 10^{-3}—10^{-6} and egg white inhibitor diluted to give a final titre 1/64 as substrate.

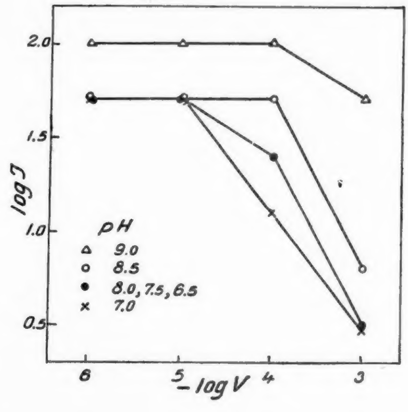


Fig. 5. — Graph showing effect of pH on the inactivation of egg white inhibitor by low concentrations of influenza virus A/F/51 in broth during 24 hours at +37°C.

I = Percentage of residual inhibitor
V = Virus dilution

The results of this test are shown in fig. 5. The best picture of the optimal pH range is given by virus dilution 10^{-4}, in which the difference was most distinct. Inactivation of the inhibitor was greatest at pH 7.0, and it declined on proceeding from this optimum toward the alkaline or the acid side.

Since pH 7.0 was thus found to be the optimum, the various tests in the present investigation were carried out with broth having this pH value.

EFFECT OF TEMPERATURE

Most studies on viral enzyme have been carried out at the temperature of + 36—37°C, which has been found favourable for

reactions of the influenza viral enzyme (16). To elucidate the significance of temperature, the capacity of small amounts of influenza virus A/F/51 to destroy the inhibitor at + 19°, + 28°, + 37° and + 39°C was studied. The experiments were made in broth by the usual method (page 31) using the same virus dilution series of 10^{-3}—10^{-6} and egg white inhibitor diluted to give a final titre 1/64 as the substrate.

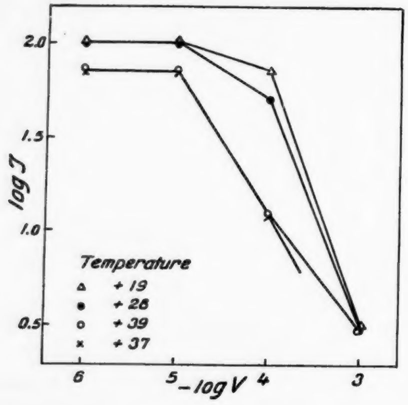


Fig. 6. — Graph showing effect of temperature on the inactivation of egg white inhibitor by low concentrations of influenza virus A/F/51 in broth, pH 7.0, during 24 hours. Temperatures tested: +19°, +28°, +37° and +39°C.
I = Percentage of residual inhibitor
V = Virus dilution

Fig. 6 shows the results obtained in these tests. The least inactivation of the inhibitor was seen at + 19°C and the most at + 37°C, although at + 39°C it was nearly as great as at + 37°C. The difference in the effect of the temperature was most evident in the virus dilution 10^{-4}, but in the higher virus concentration (10^{-3}) the inhibitor was destroyed to a great extent already at a low temperature (+ 19°C).

Of the temperatures tested, the optimal result was obtained at + 37°C, and this temperature was therefore used in also the other tests in this investigation.

RATE OF REACTION

In studies on influenza virus enzyme, comparatively large amounts of virus — several agglutinating units — have generally

been employed, as they have been found to inactivate suitable amounts of substrate rapidly, *i.e.*, in *c.* 1—2 hours (16).

In the present investigation using small virus amounts and attempting to demonstrate as small amounts as possible, it was necessary to find the shortest time during which demonstrable destruction of the inhibitor occurs. It is obvious that when low concentrations of virus are employed, the rate of inactivation is slow and a long reaction time is therefore necessary.

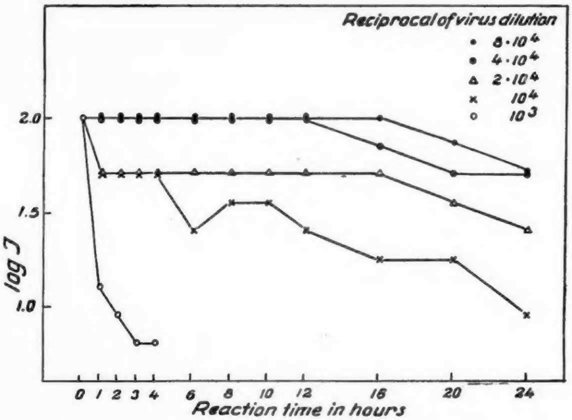


Fig. 7. — Graph showing rate of inactivation of egg white inhibitor by low concentrations of influenza virus A/F/51 in broth, pH 7.0, at +37°C.

I = Percentage of residual inhibitor

Test Technique. — Serial dilutions were made of influenza virus A/F/51 in broth, the final dilutions being 10^{-3}, 10^{-4}, 0.5×10^{-4}, 0.25×10^{-4}, and 0.125×10^{-4}. The egg white inhibitor was also diluted in broth to give a final titre 1/64. The test was carried out by the usual technique (page 31). A separate series of dilutions was prepared for each reaction time, *i.e.*, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 och 24 hours.

Fig. 7 shows the results, from which it is seen that virus dilution 10^{-3} was capable of inactivating the greater part ($\geq$ 87.5 per cent) of the inhibitor rapidly, *i.e.*, already in one hour. A marked retardation of the reaction occurred during the following hours. Dilutions 10^{-4} and 0.5×10^{-4} also effected a rapid initial inactivation of the inhibitor, followed by gradual retardation. However, this inactivation continued through the entire 24 hours, this time being required before the activity was demonstrably destroyed by dilution 0.125×10^{-4}.

EFFECT OF SUBSTRATE CONCENTRATION

In his studies on the effect of the standard amount of virus on various substrate concentrations, Burnet (16) observed that decomposition in all substrate concentrations occurred as a logarithmic function of time. However, the reaction was slower with higher concentrations, and he suggested that some factor which retards the reaction was present in high concentrations of substrate. It is therefore logical to assume that for smaller amounts of substrate a smaller amount of virus is required for inactivation of the inhibitor to be demonstrated. However, the method of measurement used sets certain limits upon the amount.

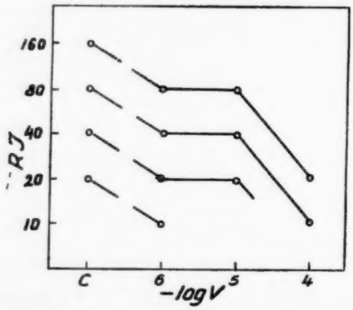


Fig. 8. — Graph showing effect of substrate concentrations within a practicable range on the enzymatic reaction of low concentrations of influenza virus A/F/51 in broth, pH 7.0, during 24 hours at +37°C, using egg white inhibitor as substrate.

RI = Reciprocal of inhibition titre
V = Virus dilution
C = Initial inhibition titre

Test Technique. — To study this question a 10-fold dilution series was made with influenza virus A/F/51 in broth, in the final dilutions of 10^{-4}—10^{-6}. Egg white inhibitor was also diluted in broth in ratios giving in the tests the inhibition titres of 1/160, 1/80, 1/40 and 1/20. A reaction time of 24 hours at a temperature of + 37°C was used. All four dilutions of the inhibitor were treated by different virus dilutions in usual manner (page 31).

The curves in fig. 8 show that virus dilutions 10^{-6} and 10^{-5} produced a 50 per cent drop in the inhibition titre of the substrate, irrespective of the concentration. Virus dilution 10^{-4} also reduced the inhibition titres by equally large percentages in two substrate concentrations.

EFFECT OF CERTAIN IONS AND ION CONCENTRATIONS

Ions have been found to be one of the most important factors affecting the course of enzymatic reactions. Thus it has been demonstrated that they are indispensable also for reactions of the influenza virus enzyme. Ions, of which Na and Ca have proved to be important, are required in a given concentration in the various functions of the influenza virus (21). Water-soluble meconium inhibitor has made the study of ion concentrations possible. Studies with viral enzyme have usually been made in physiological saline solution containing $CaCl_2$.

In the present investigation, the effect of ions on the inactivation of inhibitor by small amounts of virus was studied using solutions of NaCl, $CaCl_2$ and $MgCl_2$ and meconium and egg white inhibitors as substrate.

Test Technique. — Solutions of NaCl, $CaCl_2$ and $MgCl_2$ were prepared in distilled water in dilutions M/5, M/10, M/20, M/50 and M/100. An M/6.3 (isotonic) solution of NaCl was also made. Serial dilutions of virus were made into each of these solutions to give final dilutions of 10^{-3}—10^{-6}. Meconium inhibitor was diluted in similar solutions to contain 0.3 mg of inhibitor per ml. The experiment was carried out in the usual manner (page 31) in all dilutions of the three salts used. A similar experiment was made with egg white inhibitor (titre 1/64) in a M/6.3 solution of NaCl and a M/10 solution of $MgCl_2$. Solutions of $CaCl_2$ could not be employed in this case, since the substrate was dissolved in phosphate-buffered saline, in which the calcium formed a precipitate.

The results of the experiments are shown in fig. 9 and 10. The viral enzyme was active in each of the three salt solutions. In NaCl and $MgCl_2$ solutions of equal concentration (M/6.3—M/10) the virus showed fairly great activity over a comparatively restricted range. A fall occurred in the activity on proceeding toward either higher or lower concentrations from the optimum area. The optimum concentration range of the $CaCl_2$ solution seemed to lie at about M/10 and M/20. According to the results obtained, all the studied ions appeared to be of approximately equal value when the optimum concentration was employed.

For greater clarity of the results, the curves in fig. 9 are drawn from the experiments with virus dilution 10^{-3}, in which the destruction of substrate was greatest and the differences between the various concentrations were most marked. Approximately the same

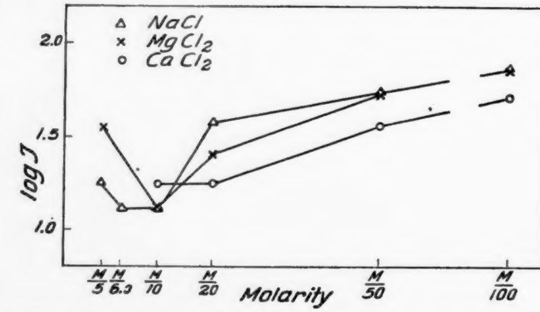


Fig. 9. — Graph showing inactivation of meconium inhibitor by influenza virus A/F/51 diluted 10^{-3}, in various concentrations of NaCl, $CaCl_2$ and $MgCl_2$ solutions during 24 hours at +37°C. Titrations carried out with Lee indicator virus.

I = Percentage of residual inhibitor

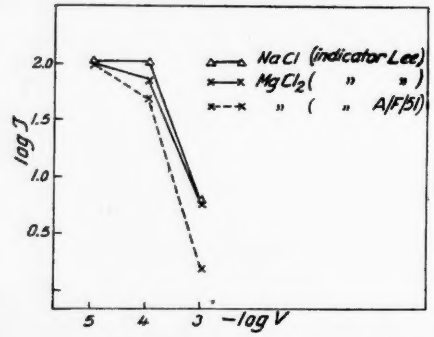


Fig. 10. — Graph showing inactivation of egg white inhibitor by low concentrations of influenza virus A/F/51 in M/6.3 solution of NaCl and M/10 solution of $MgCl_2$ during 24 hours at +37°C.

I = Percentage of residual inhibitor
V = Virus dilution

type of curves were obtained with the higher dilutions of virus (10^{-4} and 10^{-5}).

Fig. 10 shows the results obtained with egg white inhibitor in NaCl and $MgCl_2$ solutions. The ability of the influenza virus to destroy also egg white inhibitor in both solutions is evident from these results. The results obtained in $MgCl_2$ solution with different indicator viruses diverge from those obtained later in broth and saline (page 46) in the respect that in the former a greater inactivation of egg white inhibitor was demonstrated by A/F/51 indicator virus than by Lee indicator virus.

EFFECT OF ANTISERUMS

The receptor and inhibitor gradient phenomena point to the possibility of some degree of difference in the enzyme(s) of various influenza viruses (page 19).

It seemed probable that a study of the specific capacity of antiserums to inhibit the action of the homologous virus against the inhibitor would not throw much light on this subject, since the antiserum has a wide action which extends also to properties of the virus other than the enzymatic activity. However, a study of the effect of antiserums was important with a view to the latter part of the present investigation. Experiments were therefore carried out to determine the capacity of influenza A/F/51 antiserum and influenza Lee antiserum to counteract inactivation of egg white inhibitor by the corresponding viruses.

Test Technique. — Influenza A/F/51 and Lee viruses were diluted in broth to give final dilutions of 10^{-3}, 10^{-4} and 10^{-5}. Influenza A/F/51 antiserum (titre 1/320) and influenza Lee antiserum (titre 1/640), both of which had been treated with *V. cholerae* filtrate, were also diluted with broth to give final dilutions of 1/50, 1/500 and 1/5,000. A dilution of egg white inhibitor was prepared with broth to give a final titre of 1/64.

A test series was made of each virus dilution by preparing a tube for each homologous antiserum dilution as well as for the heterologous antiserum in dilution 1/50. Tubes containing no antiserum and control tubes containing inhibitor but no virus or antiserum were also set up.

The different components were combined by placing 0.5 ml of virus dilution into each test tube and adding 0.1 ml of antiserum dilution, followed immediately by the addition of 0.4 ml of inhibitor dilution. The test was continued in the usual manner (page 31). Twenty-four hours at + 37°C were allowed for reaction. The inhibition titres of the reaction solutions were determined by titration against the indicator virus after the antiserum had been destroyed by keeping the tubes in water at + 100°C for 2 minutes; this treatment was found adequate for the present purpose.

It will be seen from fig. 11 that the antiserums had a definite effect on the reaction. Dilutions of homologous antiserums as high as 1/5,000 were still capable of completely inhibiting the inactivation of inhibitor by Lee virus dilution 10^{-3} and to a great extent by A/F/51 virus in the same dilution. On the other hand, even a 100-fold greater amount of heterologous antiserum (dilution 1/50) did not prevent the inactivation of inhibitor even by the highest virus dilution used (10^{-5}).

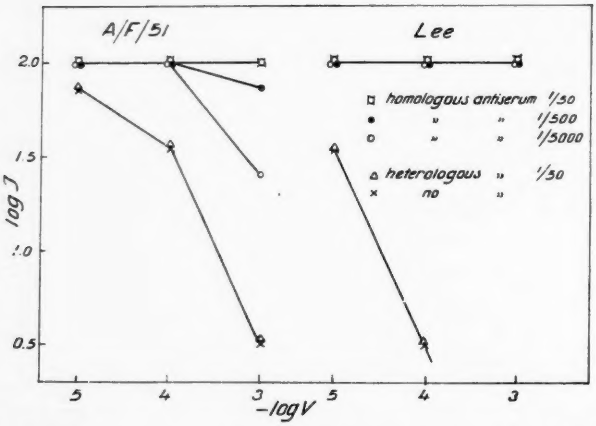


Fig. 11. — Graph showing capacity of influenza A/F/51 and Lee antiserums to prevent the inactivation of egg white inhibitor by the same viruses in broth, pH 7.0, during 24 hours at +37°C.

I = Percentage of residual inhibitor
V = Virus dilution

ENZYMATIC ACTIVITY OF FRESHLY ISOLATED STRAINS OF INFLUENZA VIRUS A/F/53

It had been demonstrated in studies earlier referred to (119) that influenza viruses in O phase were capable of enzymatically destroying human inhibitor but not avian inhibitor. Indicator viruses in O phase are likewise inhibited by human mucoid only. Since viruses isolated from patients with influenza have been found to be in the O phase immediately after isolation (6), it was assumed that human meconium inhibitor is a more suitable substrate than egg white inhibitor for viruses from the throat washings of influenza patients. This subject was studied by using virus strains isolated from four patients during the influenza epidemic in Finland in 1953 and passed through one amnion (in one case two amnion) and one allantois passage. Even at high titres these virus strains agglutinated chicken red cells already after the first egg passage. Thus they were not O phase viruses in the proper sense of the word, although the strains had recently been isolated from human patients.

Test Technique. — Determinations were made of the enzymic activity of the following freshly isolated virus strains: A/F/15/53/E2 (1 amnion and 1 allantois passage, haemagglutination titre for chicken red cells 1/1,024),

A/F/16/53/E2 (1 amnion and 1 allantois, 1/512), A/F/21/53/E2 (1 amnion and 1 allantois, 1/512), and A/F/20/53/E3 (2 amnion and 1 allantois, 1/1,024). Serial dilutions with broth were made of each virus to give final dilutions of 10^{-3}—10^{-6}. The egg white inhibitor was diluted with broth to give a final inhibitor titre of 1/64, and the meconium inhibitor was diluted in the ratio 0.3 mg/ml. The test was carried out in the usual manner (page 31). The titrations were performed against two indicator viruses and the results are shown in fig. 12.

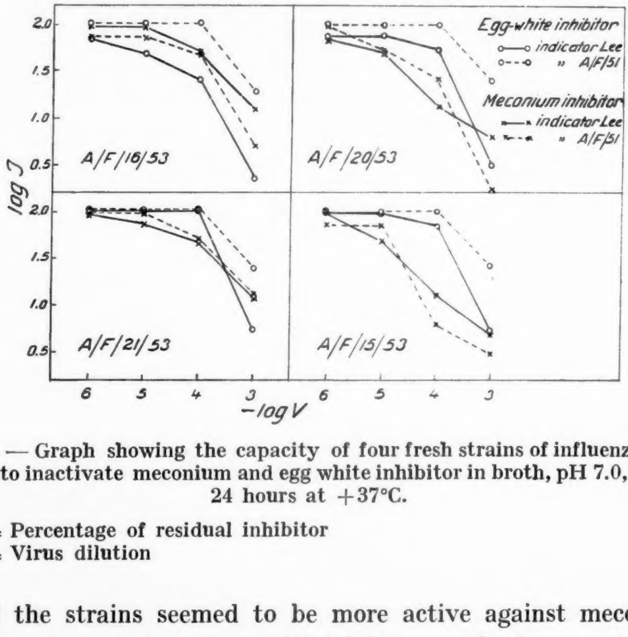


Fig. 12. — Graph showing the capacity of four fresh strains of influenza virus A/F/53 to inactivate meconium and egg white inhibitor in broth, pH 7.0, during 24 hours at +37°C.

I = Percentage of residual inhibitor
V = Virus dilution

All the strains seemed to be more active against meconium inhibitor than against egg white inhibitor, with the exception of A/F/16/53 and A/F/21/53 (the latter in dilution 10^{-3} and in titrations with Lee indicator virus).

The Lee indicator virus was remarkably more »sensitive» than A/F/51 virus as an indicator of the inactivation of egg white inhibitor. For meconium inhibitor, on the other hand, the two indicator viruses gave approximately similar results, in contradiction to the results obtained with meconium inhibitor in later experiments (page 46).

These findings seem to indicate that human meconium inhibitor may be more suitable for use as substrate than egg white inhibitor when fresh human influenza viruses of this kind are to be demonstrated by means of the enzymic property of the virus.

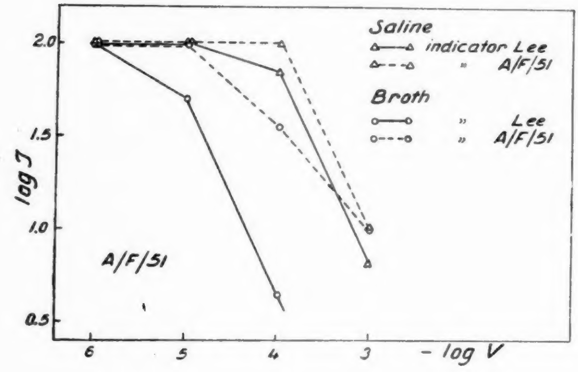
STUDIES RELATING TO THE INHIBITOR GRADIENT

In studies reported in the literature the inhibitor gradient phenomenon has been found to be present only in cases in which the virus is allowed to act on the inhibitor for a short time only, but if the reaction time is long the virus receptors are destroyed (74). Inactivation of inhibitor takes place slowly with small amounts of virus, and it therefore appeared probable that the initial stage of inactivation, and therefore also the inhibitor gradient, would be clearly demonstrable. This occurrence was studied with A/F/51 and Lee viruses in broth and solutions of NaCl, $CaCl_2$ and $MgCl_2$, using egg white inhibitor and meconium inhibitor as substrates.

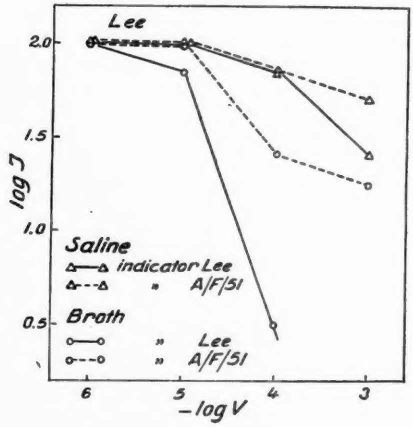
Test Technique. — Serial dilutions of influenza Lee virus were made with broth and isotonic NaCl solution and A/F/51 virus also with M/10 solutions of $CaCl_2$ and $MgCl_2$ in ratios to give final dilutions of 10^{-3}—10^{-6}. The egg white inhibitor was diluted with broth and NaCl solution to give the final titre of 1/64. Meconium inhibitor was likewise diluted in the ratio of 0.3 mg/ml with isotonic NaCl solution and M/10 solutions of $CaCl_2$ and $MgCl_2$ to give final concentrations of 0.3 mg/ml. The experiments with egg white inhibitor were performed as usually (page 31), using both viruses in broth and NaCl solution. The meconium inhibitor was treated with A/F/51 virus in the three salt solutions and with Lee virus in NaCl solution only. The titrations were carried out with both indicator viruses.

The results are given in figs. 13 A, 13 B, 14 A, and 14 B. Both viruses effected a greater inactivation of egg white inhibitor in broth than in NaCl solution. The inhibitor gradient is clearly apparent in the graphs. In all the tests with egg white inhibitor, titration against Lee indicator virus indicated a greater inactivation of inhibitor than when the titrations were made with A/F/51 indicator virus. Thus, quite independently of which virus was used for destruction of inhibitor, Lee indicator virus was able to demonstrate in broth the inactivation effected by virus dilution 10^{-5}, whereas with A/F/51 the destruction of inhibitor could be demonstrated in virus dilution 10^{-4}. This relative order of demonstration by the two indicator viruses was similar in both broth and saline.

A/F/51 virus had an approximately equal activity against meconium inhibitor in the three salt solutions. Contrary to what was the case with egg white inhibitor, A/F/51 indicator virus was



A

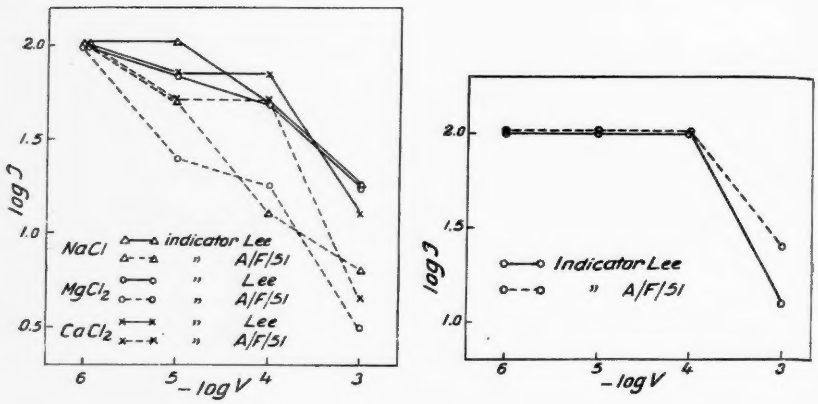


B

Fig. 13 A and B. — Graphs showing loss of activity for A/F/51 and Lee indicator viruses of egg white inhibitor treated with low concentrations of influenza A/F/51 (A) and Lee (B) viruses during 24 hours at +37°C in broth, pH 7.0, and saline.

I = Percentage of residual inhibitor
V = Virus dilution

a much more »sensitive» indicator of the inactivation of meconium inhibitor than the Lee indicator virus. A/F/51 indicator virus was capable of demonstrating the inactivation of meconium inhibitor by virus dilution 10^{-5}, which corresponds to the inactivation of egg white inhibitor in broth shown by Lee indicator virus. It was earlier seen (page 33) that A/F/51 virus showed but little difference



A B

Fig. 14 A. — Graph showing loss of activity for A/F/51 and Lee indicator viruses of meconium inhibitor treated with low concentrations of influenza A/F/51 virus in M/6.3 solution of NaCl and M/10 solutions of $CaCl_2$ and $MgCl_2$ during 24 hours at +37°C.

Fig. 14 B. — Graph showing loss of activity for A/F/51 and Lee indicator viruses of meconium inhibitor treated with low centrations of Lee virus in M/6.3 solution of NaCl during 24 hours at +37°C.

I = Percentage of residual inhibitor
V = Virus dilution

in the activity against meconium inhibitor in saline and in broth when titrated against Lee indicator virus. The Lee virus destroyed but poorly the meconium inhibitor in NaCl solution, as destruction of inhibitor was seen in virus dilution 10^{-3} only. It would seem that in this case Lee was a more »sensitive» indicator virus than A/F/51, although the difference is not great.

Effect on the Inhibitor Gradient of Chicken Red Cells Used in Titration

In studying the destruction of inhibitor the various effects of the destroying virus, substrate and indicator virus on the results was usually taken into consideration. A further factor — the chicken red cells — takes part in the reaction in the titration procedure and its effect is associated with the same mechanism as that of the above mentioned factors.

Chicken red cells have been found to be so variable in their properties that up to 500-fold differences have been obtained with

meconium inhibitor by using red cells from different chickens (105). It is therefore important to take this factor into consideration in experiments with inhibitor. Chicken red cells do not seem to be as variable when used with egg white inhibitor as they are with meconium inhibitor (105).

In the system used for titration of the activity of the inhibitor there is an affinity between the indicator virus and the inhibitor,

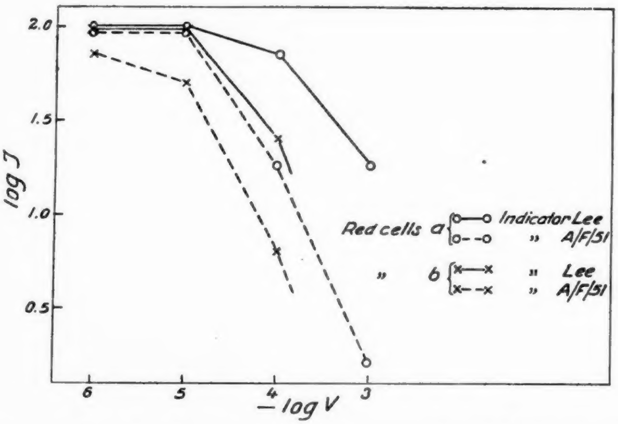


Fig. 15. — Graph showing inactivation of meconium inhibitor by low concentrations of A/F/51 virus in broth, pH 7.0, during 24 hours at +37°C when titrated with A/F/51 and Lee indicator viruses, using two kinds of chicken red cells having different sensitivities for meconium inhibitor.

I = Percentage of residual inhibitor
V = Virus dilution

on the one hand, and the indicator virus and the red cells, on the other hand. When red cells which behave differently in this respect are employed in the titrations, diverging inhibitor titres will be obtained. If the inhibitor is destroyed, it seems likely that the mutual affinities in the inhibitor — indicator virus — red cell system are changed and the use of red cells of different sensitivities will show inhibitor destruction of different degrees.

To study this phenomenon, meconium inhibitor was treated with small amounts of A/F/51 virus in the usual manner (page 31) and titrations were made against A/F/51 and Lee indicator viruses using chicken red cells of two kinds, which differed in their sensitivity to meconium inhibitor.

Test Technique. — A dilution series of influenza A/F/51 virus was made with broth to give final dilutions of 10^{-3}—10^{-6}. Meconium inhibitor was diluted with broth to contain inhibitor in the ratio of 0.3 mg/ml. The test proceeded as usual (page 31). The titrations were made against both A/F/51 and Lee indicator viruses, using for each virus two kinds of chicken red cells of different sensitivities for meconium inhibitor giving the titres of 1/320 and 1/2,560 with Lee indicator virus.

The results of the tests are shown in fig. 15. As in the preceding test (page 46) the A/F/51 indicator virus demonstrated a greater inactivation of meconium inhibitor than the Lee indicator virus. By employing the chicken red cells of different sensitivities for meconium inhibitor, definitely diverging results were obtained in titrations with the same indicator virus also, although, for technical reasons, cells which would have given maximum differences were not selected. This »chicken cell gradient» was brought out by both of the indicator viruses. In this way a double gradient was obtained.

DISCUSSION

It was the effort in this investigation to study as many factors as possible which influence the reaction in question. The field of investigation was therefore a relatively large one.

Broth was used as the reaction medium in most of the tests. Different batches of broth may vary greatly and it is difficult to determine their exact composition. It was found that all the batches of broth were not equally suitable for the purpose, but they were in all cases superior to saline.

It is not known why the virus enzyme is capable of inactivating egg white inhibitor in broth better than in NaCl solution. One of the differences between these media is the protein contained in broth, which is known to afford protection to viruses. The addition of human albumin, but not of peptone, to saline converted it to a medium corresponding to broth (104). It may therefore be assumed that the superiority of broth as a medium is due to the circumstance that the small virus amounts used in these tests maintain their activity better in broth than in saline during the long reaction time. This surmise is supported by the observation made earlier in our laboratory that the drop in the infection titre of the virus in saline during incubation at + 37°C for 24 hours was 1,000-fold as compared to the decline in broth (104). How-

ever, no notable reduction of enzymic activity was observed at the same time. In the present investigation, the use of virus in amounts larger than one agglutinating unit did not demonstrate definite inactivation of the viral enzyme either in saline or broth during the reaction time used.

It might be assumed that the diverging results obtained in the different media are due to the possibly better elimination in broth than in saline of the possible destruction products formed in, and inhibiting, the reaction. However, it has not been possible to observe the production of such reaction products (16). In addition to proteins, broth also contains the various componeets of meat extract, the influence of which on the reaction cannot readily be studied.

In broth, A/F/51 virus dilution 10^{-5} was found to have produced a *c.* 50 per cent inactivation of the egg white inhibitor when titrated against Lee indicator virus, whereas in saline a corresponding inactivation was seen in dilution 10^{-3}. The haemagglutination titres of the viruses studied were 1/2,048 and 1/4,096, and thus it was possible to demonstrate in broth, by means of the enzymic activity, virus concentrations which were *c.* 25—50 times lower than the concentrations demonstrable with the aid of haemagglutination. In addition to haemagglutination and infection, a suitable means is therefore available for the demonstration of small amounts of influenza virus. The sensitivity of this method based on the inactivation of inhibitor lies between those of the two above mentioned reactions.

The finding that $+ 37°C$ is the optimum of the temperatures tested in this study is in agreement with earlier observations reported in the literature (16). The enzyme is fairly active also at lower temperatures, so that differences were obtained at various temperatures with virus dilution 10^{-4} only. This is evidence indicating that the use of small amounts of virus makes it possible to demonstrate the effect of factors which have a weak action only. The result obtained in this respect shows that small changes occurring in the temperature during the reaction have very little effect on the course of the reaction. This is also true of changes in the pH of the reaction medium, the tested virus being active over a comparatively wide pH range.

In studying the enzymic activity of small amounts of virus it is necessary to use substrate amounts which are of a given order

of magnitude. Since with the haemagglutination technique used in this investigation it is possible to demonstrate only 50 per cent and greater inactivation, the amount of substrate must be small in order to effect inactivation with as small virus amounts as possible. On the other hand, the amount of substrate should be sufficiently large to permit the inhibition of haemagglutination in the inhibition test. In other words, the concentration of substrate should be so high that inhibition is effected, or preferably several times as high as this concentration in order that also over 50 per cent destruction of substrate, may be demonstrated. According to the results obtained, there is no alteration in the sensitivity of the reaction even if the substrate concentration varies over the range of titres 1/160—1/20 when the inactivation of the inhibitor is measured by the method here used.

When large amounts of virus and a short reaction time are employed, the inactivation of the substrate by active viruses is a logarithmic function of time (16). In the presence of small amounts of virus the destruction was at first logarithmic, but as the reaction proceeded it was progressively retarded when the destruction of substrate increased, as usually is the case in enzymic reactions.

The results agree with those reported in the literature in the respect that those obtained with virus dilution 10^{-3} correspond to the rate of reaction of highly active viruses and the values obtained with higher dilutions of virus correspond to the reaction rate of less active viruses.

Since with the smallest amounts of virus the destruction of the inhibitor occurs through 24 hours, this time, if possible, should be used in order to demonstrate small amounts of virus by means of their enzymatic properties. Thus it is possible to produce an inactivation which is two to three times as great as that effected during a reaction time of one hour.

Since the Mg ion is known to activate also several other enzymes, it is not an unexpected finding that $MgCl_2$ solution in the same molar concentrations as $CaCl_2$ and NaCl proved an equally suitable medium for the viral enzyme reaction. According to the present investigation, the Ca ion does not appear to be specific for viral enzyme activity, though it may be possible that the allantoic fluid virus, although greatly diluted, may already contain an adequate amount of Ca ions (35). On the contrary, the destruction of inhibitor

by the virus occurs in practically the same manner in NaCl, $CaCl_2$ and $MgCl_2$ solutions. With regard to the ionic concentrations the results obtained are in agreement with those reported in the literature for large amounts of virus (21).

Both of the homologous antiserums employed prevented the destruction of inhibitor in a *c.* 10-fold greater serum dilution than that in which haemagglutination was inhibited, although approximately equal amounts of virus were used in the two tests. It is not possible to determine on the basis of the present tests whether this was due to the antiserum possessing here also specific antienzymic properties or, for instance, merely to the longer time allowed for action of the antiserum in this test than in the haemagglutination inhibition test. That an antienzymic property may have been effective here is suggested by the observation that the homologous antiserum in dilution 1/5,000 prevented the effect on the inhibitor of a 100-fold greater virus amount than that against which the heterologous antiserum diluted 1/50 was entirely ineffective. In any case it is indicated by this investigation that in tests to demonstrate the influenza virus by means of its capacity to destroy inhibitor it is possible to employ antiserums to determine the type of virus responsible for the destruction. Should it be possible to specifically differentiate also subtypes of influenza A and B viruses by means of antiserums, we would have an important aid to the identification of viruses.

Strains of influenza virus A/F/53 isolated in Finland during an epidemic in 1953 were capable, after two or three egg passages, to agglutinate not only human red cells but also chicken red cells. For this reason it was presumed that they would also be enzymatically active against inhibitor of both human and avian origin. However, most strains seemed to be more active against human inhibitor (meconium) than against egg white inhibitor. The enzymic activity of these freshly isolated strains against meconium inhibitor was, in comparison to their haemagglutinative capacity, considerably greater than that of the earlier tested A/F/51 virus which had passed through 25—34 allantois passages and was somewhat more active against egg white inhibitor than against meconium inhibitor. The results, it is true, were dependent on the indicator virus used. Thus, for example, strain A/F/16/53 inactivated the substrate in virus concentrations that were 200 times lower than the

concentration which effected haemagglutination. This observation offers a possibility to demonstrate influenza virus in the throat washings.

When small amounts of virus were employed the receptor gradient phenomenon was clearly observable. It is important to take this gradient into consideration in seeking to demonstrate the effect of small amounts of virus, for which an indicator virus of the highest possible sensitivity is necessary. The transposed order of the curves obtained for the destruction of egg white inhibitor by different indicator viruses in $MgCl_2$ solution (page 42), as compared with those obtained in NaCl solution (page 46), seems to point to the possible influence of the reaction medium on the order of inactivation. The order obtained with freshly isolated strains of virus was similar to that obtained with strain A/F/51 in tests with egg white inhibitor, but different from that in tests with meconium inhibitor.

The test performed with meconium inhibitor and chicken red cells of various degrees of sensitivity indicates the complicated character of the inhibitor gradient phenomenon. The inhibitor gradients obtained in previous studies reported in the literature might not wholly, at least, be an indication that in the tests the receptors for different viruses in the inhibitor had undergone destruction to a different extent or in a different order. The results in those studies were probably influenced also by the use, in the titrations, of indicator viruses possessing varying degrees of affinity for red cells. Diverging results for inhibitor destruction were obtained by the use of one indicator virus but two kinds of red cells of different sensitivity. Analogously, the use of two indicator viruses with possibly varying affinities for the same red cells will not give fully correct information on the destruction of those receptors in the inhibitor which correspond to these viruses. This may possibly provide an explanation also for the slight variations in the inhibitor gradient curve (17, 74). This phenomenon, however, is clearly seen only with inhibitors of the meconium inhibitor type, which apparently have a poor affinity for indicator viruses. Theoretically considered, every indicator virus used in inhibitor gradient studies should have specific red cells in order that the affinity of each virus — red cell combination in the titrations would be identical.

The temperature and pH used in the first tests in broth proved to be the optimal conditions. Likewise a reaction time of 24 hours and the substrate concentrations used seem suitable for the demonstration of small amounts of influenza virus. No marked difference was observed in the results obtained in broth and saline when meconium inhibitor was used.

Special attention should be paid to the origin of the substrate, to the properties of the indicator virus, and to the type of antiserum used. If an unknown virus is to be demonstrated it is difficult to specify beforehand the most suitable substrate and indicator virus to be employed. In titrations of meconium inhibitor, the kind of red cells should also be taken into consideration.

EXPERIMENTS TO DEMONSTRATE INFLUENZA VIRUSES FROM THROAT WASHINGS

In the experiments described in the preceding chapter it was found that with the aid of the enzymic property of the influenza virus it is possible to demonstrate virus amounts which have an enzymatic activity corresponding to allantoic virus in *c.* 10^{-5} dilution. It was observed, furthermore, that freshly isolated strains possess a high enzymatic activity in comparison to their haemagglutinating capacity. The experiments also showed that inactivation of the inhibitor can be specifically counteracted by antiserum.

The »sensitivity» of the reaction between the virus and the inhibitor substrate is of at least the same order of magnitude as the highest virus concentrations in the throat washings of patients with influenza. Virus concentrations as high as 10^7 chick embryo infection doses per cc have been found in such throat washings (76). It was therefore believed that demonstration of the influenza virus should be possible directly from throat washings. This possibility was augmented by the ability to employ red cell adsorption, which will yield an approximately 8-fold increase of the original concentration and eliminate the throat washing fluid.

For this purpose, experiments were carried out with a number of throat washings from patients with influenza and respiratory

infections and from healthy persons. Although the series studied were small, the results are presented for their possible orientative value.

MATERIAL AND METHODS

Three series of throat washings were examined. The samples in group I were collected during an epidemic of influenza A in Finland in February—March 1953 from patients ill with symptoms of influenza in the febrile stage. Group II comprised samples taken in the autumn of 1953 from persons with infections of the respiratory tract, and group III were samples collected at the same time as those in group II from healthy persons who had not suffered from respiratory infections during the preceding two weeks.

After development of the method, which consumed a large number of samples, 11 samples from influenza patients were examined in the spring of 1953. However, the exact statistical analysis of the results does not include cases in which the value obtained in one or more of the three parallel titrations of the sample fell below or above the range of measurement so that the exact titre could not be determined. When examinations were resumed in the autumn, by which time the remaining influenza samples had been stored for about 3—5 months at — 18°C, only 3 of the 25 samples then examined gave results analogous to those obtained in the preceding spring, because of inability to maintain a sufficiently low storage temperature. In none of these 25 cases was culture of the virus in the egg possible after storage. Thus the results obtained in the autumn did not correspond to those obtained from samples tested immediately and they therefore were not taken into consideration. The samples from persons with respiratory infection and from healthy subjects were tested not later than one month after taking, 23 samples from the former and 15 samples from the latter being examined. All values, however, were not included in the exact statistical analysis of results for the reason mentioned above.

Test Technique. — To each throat washing fluid stored at — 18°C (*c.* 10 ml of broth, a part of which had been lost during gargling) was added a sufficient amount of broth to make 12 ml. The samples were shaken and cetrifuged at 2,000 r.p.m. for 15 minutes and the supernatant fluid was distributed into three test tubes containing 4 ml each. To each tube was added 0.5 ml of 0.5 per cent chicken red cells for adsorption of the viruses, and the whole was shaken and allowed to stand for 15 minutes at room temperature (*c.* + 18°C) and for two hours at +4°C, with occasional shaking. The cells were then centrifuged down at 1,500 r.p.m. for 5 minutes at + 4°C and washed twice at + 4°C with saline of the same temperature. Over the washed cells was added 0.1 ml of broth

to the first tube, 0.1 ml of influenza A/F/53 antiserum (titre 1/320) diluted 1/15 to the second tube, and 0.1 ml of influenza Lee antiserum in the same dilution to the third tube. The tubes were shaken and allowed to stand at room temperature for 2 minutes. After the addition of 0.4 ml of egg white diluted 1/100 and containing 500 units of penicillin and 500 γ of streptomycin per millilitre, the tubes were shaken, closed with a stopper and placed in the water bath at + 37°C for 24 hours. The tubes were shaken from time to time when possible.

After incubation for 24 hours the contents of the tubes were centrifuged once more at 1,500 r.p.m. for 5 minutes and the supernatant was removed with a Pasteur pipette. Before titration of the residual inhibitor the reaction solution was kept in water at + 100°C for 2 minutes to destroy the antiserum.

The inhibition titre obtained was compared with the titre of a control tube containing the corresponding dilution of inhibitor under the same test conditions. In some cases an additional control was used in which broth alone was adsorbed with red cells and the inhibitor dilution corresponding to the test was added, after which incubation was carried out under identical conditions. Several tests using this control gave results that were similar to those obtained with the first mentioned control.

STATISTICAL ANALYSIS METHOD

The results were analysed statistically by the following method.

Let x_i $(i = 1 \cdots n)$ be the series of observed values. As measure of the average level shall be regarded the arithmetic mean of the series of observed values

$$\bar{x} = \frac{1}{n} \sum x_i$$

The reliability of this mean value is indicated by its standard error or deviation of the mean $s_{\bar{x}}$, which is obtained from the following formula:

$$s_{\bar{x}} = \sqrt{\frac{1}{n\,(n-1)} \sum (x_i - \bar{x})^2}$$

The result is expressed briefly in the following formula:

$$\bar{x} \pm s_{\bar{x}}$$

On this basis it is possible to calculate the confidence limits which correspond to the probability of p %, as follows:

$$x \pm t_p s_{\bar{x}}$$

where t_p is obtained from the t table. When the confidence interval corresponding to the probability of p % is wholly outside zero, it indicates that with this probability there is a deviation from zero (28).

RESULTS

The results obtained in the tests with throat washings are shown in the graph in fig. 16.

The highest mean inactivation of the inhibitor was obtained in the group of respiratory infections (group II), in which there remained a mean residual of 8 per cent (0.90 $\pm$ 0.241, log) of the substrate. In the group of healthy persons (group III) the mean residual was 25 per cent (1.40 $\pm$ 0.164, log) and in the influenza

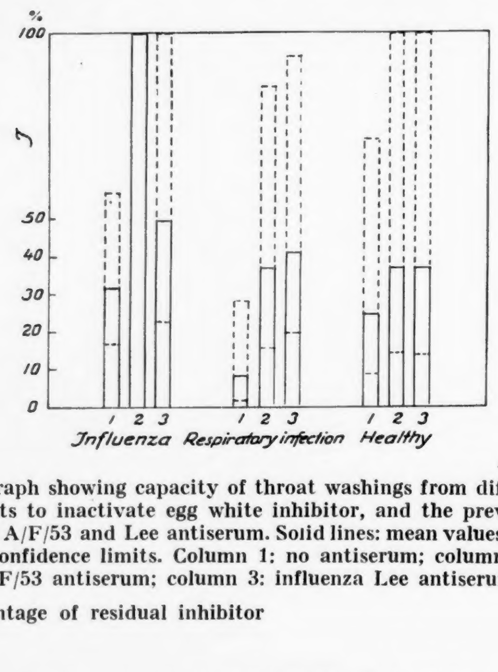


Fig. 16. — Graph showing capacity of throat washings from different groups of test subjects to inactivate egg white inhibitor, and the prevention of inactivation by A/F/53 and Lee antiserum. Solid lines: mean values; dotted lines: 95 per cent confidence limits. Column 1: no antiserum; column 2: influenza A/F/53 antiserum; column 3: influenza Lee antiserum.

I = Percentage of residual inhibitor

group (group I) 32 per cent (1.50 $\pm$ 0.100, log), the last mentioned thus showing the lowest degree of inactivation.

The A/F/53 antiserum completely inhibited the destruction of inhibitor in the influenza group (group I). The difference between columns 2 and 1, *i.e.*, between the results of tests with A/F/53 antiserum and with no antiserum, was 0.50 $\pm$ 0.100, log, which is statistically significant. In group II this difference was 0.56$\pm$ 0.226, log, which also is statistically significant, and in group III 0.18 $\pm$ 0.073, log, which is not significant. Thus the A/F/53 antiserum inhibited the reactions to approximately the same degree

in the influenza group and in the respiratory infection group, but to a lesser degree in the group of healthy persons.

The difference between columns 3 and 1, *i.e.*, between the results of tests with Lee antiserum and with no antiserum, was 0.63 ± 0.214, log in group II, which is statistically significant. The differences of 0.20 ± 0.100, log in group I and 0.18 ± 0.073, log in group III are not significant. In the group of respiratory infections (group II) the difference between columns 3 and 1 is slightly greater than the difference between columns 2 and 1. Accordingly in this group the inactivation of inhibitor was also prevented by the Lee antiserum, and even to a slightly greater degree than by the A/F/53 antiserum.

In addition to this finding it was observed that the Lee antiserum inhibited the reaction in group III to the same extent as the A/F/53 antiserum. Thus the difference between columns 2 and 3 in groups II and III is nearly the same, being negative in group II and O in group III. Only in the influenza group is there clearly a difference in the inhibition obtained with the two antiserums. Thus the difference between columns 2 and 3 in this group is 0.30 ± 0.134, log; however, owing to the small number of cases this result is not statistically significant. If the results were evaluated only according to the mean values, the inhibition caused by the A/F/53 antiserum would be twice as great as that produced by the corresponding Lee antiserum, and there would be no difference in the other groups.

No definite conclusions regarding the diagnostic significance of a test of this type from throat washings may be drawn from the present results, although they may serve as an indication of the serviceability of such a test. On the basis of the results obtained in these tests it would appear that: 1) Not only in persons with a respiratory infection but evidently also in healthy persons — at least during a respiratory disease epidemic — there is in the throat a factor which inactivates the inhibitor of viral haemagglutination and the activity of which is partially inhibited by the two kinds of influenza chicken antiserum used in these tests; 2) As a result, when the enzymatic activity of the influenza virus is utilised in a diagnostic test for influenza, the use of different influenza antiserums is necessary to give the test diagnostic value.

CAPACITY OF BACTERIA TO INACTIVATE THE VIRUS HAEMAGGLUTINATION INHIBITOR

From the foregoing experiments it appeared probable that in the throats of persons with respiratory infections as well as of healthy persons there is a factor which inactivates the inhibitor. It may be possible that this factor is produced by bacteria commonly present in the throat. It is known that filtrates of *V. cholerae* and *Cl. welchii* contain an enzyme which powerfully destroys the viral enzyme substrate (page 11). Similar observations have also been made regarding bacteria of the *Corynebacterium* and *Haemophilus* groups (34, 84) but not regarding certain intestinal bacteria (25). An enzyme with an action similar to that of RDE has also been demonstrated in human saliva (77, 82).

Bacteriological determinations were also made routinely of the above mentioned throat washings from persons with respiratory infections, using ordinary agar, blood agar, chocolate agar and semi-liquid thioglycolate agar. The bacteria isolated from the samples are listed in table 1.

TABLE 1

BACTERIA ISOLATED FROM THROAT WASHINGS OF PATIENTS WITH RESPIRATORY INFECTIONS

Bacterium	No. of Cases
Streptococcus α-haemolyticus	21
Streptococcus β-haemolyticus	3
Micrococcus pyogenes var. albus	2
Neisseria	18
Sarcina	2
No bacteria	2

From most of the specimens it was possible to isolate *α*-streptococci and *Neisseria*, which are known to be present in the throats of healthy persons also. In most of these cases both bacteria were present at the same time.

To determine the enzymatic activity of bacteria the capacity of some filtrates of the most common throat bacteria to inactivate egg white inhibitor was studied.

Test Technique. — Seven different bacteria were isolated from throat and sputum samples sent for examination to the State Serum Institute.

They were cultured in broth at + 37°C overnight, after which the bacteria were removed by centrifugation at 3,000 r.p.m. for 10 min. and discarded. The broth used as culture medium was adsorbed onto 1.0 ml of 0.5 per cent chicken red cells, as was done in the case of throat washings (page 56). The cells were washed twice at + 4°C with saline of the same temperature and centrifuged at 1,500 r.p.m. at + 4°C. Over the cells was added a substrate of 0.4 ml of filtered, diluted 1/100 and centrifuged egg white, containing the previously mentioned amounts of penicillin and streptomycin (page 31). After shaking, the tubes were closed with a stopper and placed in the water bath at + 37°C for 24 hours. The cells were then centrifuged down at 1,500 r.p.m. for 5 minutes, the inhibitor solution was removed with a Pasteur pipette, and titration was carried out against the indicator virus as described above (page 30). The results were compared with controls containing the corresponding inhibitor dilutions under the same test conditions.

TABLE 2

PERCENTAGES OF RESIDUAL EGG WHITE INHIBITOR AFTER TREATMENT WITH CHICKEN RED CELLS INTO WHICH BACTERIAL CULTURE BROTHS WERE ADSORBED

Bacterium	Percentage of Residual Inhibitor
Streptococcus α-haemolyticus	< 1.6
Streptococcus β-haemolyticus	< 1.6
Micrococcus pyogenes var. albus	<12.5
Neisseria	< 1.6
Streptococcus pneumoniae, type 3	< 3.1
Klebsiella	< 3.1
Haemophilus influenzae	25.0

The results obtained are shown in table 2. During culture in broth, all the bacteria studied produced a factor which was adsorbed onto the red cells and was capable of destroying egg white inhibitor to a considerable degree, with the exception of *H. influenzae*, which is not known to grow in broth and which had only a weak activity. After passing through the bacterial filter (Jener Ganzglasfilter 3 G 5) broth in which *α*-streptococci or *Neisseria* had been cultured behaved in a manner similar to that of broth from which the bacteria were removed by centrifugation.

DISCUSSION

During an epidemic of influenza A in Finland in 1953, isolation of the virus was possible in the chicken embryo in about 50 per cent of the samples taken from patients with symptoms of in-

fluenza (101). If the relationship between the egg infectivity and the enzymatic activity of the influenza A virus present in the throat is the same as that of the A/F/51 virus adapted to the egg, it is, according to the first part of the present investigation, possible by employing the red cell adsorption, elution and enzymatic activity of the virus to demonstrate it in those throat washings only which have an infection titre of about 10^{-3} or higher. Since a positive reaction is obtained in egg cultures in 50 per cent of the cases only, it might be assumed that the method under experimentation can give an »influenza type reaction» in a still smaller percentage of samples. It is possible, however, that the relationship between the egg infectivity and the enzymatic activity against human inhibitor is not similar in human pathogenic viruses and in egg-adapted viruses, and that the former has a greater enzymatic activity.

Further investigations of the method used in these experiments and of its diagnostic application can be made only during the occurrence of future epidemics. Although the present series of cases is small, the results obtained with the samples from influenza patients point to the probability that the factor which inactivated the inhibitor was the influenza A/F/53 virus isolated during the same epidemic, since inhibitor-destroying action was completely inhibited by A/F/53 antiserum but not by Lee antiserum. It is, of course, true that in the samples from patients with respiratory infections, A/F/53 antiserum prevented the inactivation to nearly the same extent; however, also Lee antiserum produced a similar inhibition of inactivation.

From the results obtained it would appear that when isolated samples are examined the results will be of diagnostic value only if different antiserums are used and their inhibition of inactivation is compared. It is nevertheless well to limit the number of antiserums, as the use of too many will necessitate division of the sample into impracticably minute portions.

The circumstance that samples from patients with respiratory infections and from healthy persons also caused inactivation of the inhibitor, and even to a greater degree than the samples from patients with influenza, was probably due to the production of an inhibitor-inactivating factor by bacteria present in the throat. The common throat bacteria studied were found to produce a

factor of this kind during growth *in vitro*. At the time of the respiratory infection epidemic during which the samples were taken it was not possible to isolate any influenza viruses by egg culture (102).

The inhibitor-inactivating factor found in these series of patients may also have been the factor resembling RDE which has been demonstrated in saliva. This, however, does not render it impossible that the factor may have been produced by the throat bacteria.

The factor which appeared to be produced by various throat bacteria and which possessed enzymatic activity was identical with the RDE of *V. cholerae* in the respect that it was adsorbed onto red cells and inactivated the egg white inhibitor. It may be considered possible that all the micro-organisms which multiply in the respiratory tract possess such a factor. If this is true, it forms a complication in the use of the method developed in the present investigation.

It would seem that the method here presented deserves further study to determine its diagnostic value, since, if found serviceable, it offers a simple *in vitro* method for the diagnosis of influenza in *c*. 24 hours and for the simultaneous typing of the virus concerned. In addition to the complication mentioned above, a further drawback is the, to some extent, dissimilar behaviour of different viruses, which renders it necessary to select at least the inhibitor, indicator virus and chicken red cells which are optimum for the virus type suspected of being the infective agent.

SUMMARY

Based on the observation made earlier in this laboratory that considerably smaller amounts of virus can be demonstrated by means of the enzymatic activity of the influenza virus in broth or albumin-saline solution than by its haemagglutinative activity, a study has been made of the capacity of small amounts of influenza virus to inactivate the inhibitor of viral haemagglutination. The ultimate object was the possible development of a diagnostic test for influenza based on this capacity.

The present experiments were carried out with influenza viruses A/Finland/51 and B/Lee, using egg white and meconium inhibitors. It was observed that when egg white inhibitor was used as substrate a demonstrable inactivation of the inhibitor was produced in broth by virus concentrations even 100 times lower than those required for inactivation in saline solution. By means of the enzymatic activity it thus was possible to demonstrate virus amounts 25 to 50 times smaller than by means of the haemagglutinative activity. When native egg white diluted 1/100 was used as substrate, there was only a *c.* 10-fold difference in the inactivation produced in broth and saline. This difference was still smaller when the substrate was meconium inhibitor.

In experiments on the stability of the enzymatic activity of the virus in broth and saline during a reaction time of 24 hours at + 37°C it was demonstrated that the decrease of activity was very small in both reaction media.

The optimum pH of broth for the reaction was 7.0, the reaction becoming weaker on proceeding toward the alkaline or the acid side.

Of the four temperatures tested, + 37°C was found to be optimal. Considerable inactivation of the substrate still occurred at + 18°C.

In experiments using a constant amount of inhibitor and various virus dilutions, inactivation of the substrate was produced by

virus dilution 10^{-3} at a rapid rate, *i.e.*, in 3—4 hours. With lower virus concentrations the inactivation continued through the entire test period of 24 hours, the higher of these concentrations inactivating a large proportion of inhibitor already during the first hours of reaction, whereas with the lowest concentration the inactivation was demonstrable only after the period of 24 hours.

When the influence of the substrate concentration on the sensitivity of the reaction was studied using substrate concentrations equal to titres 1/160—1/20, it was found that the virus amounts used always destroyed an equal amount of inhibitor regardless of the amount of substrate used.

Solutions of NaCl, $CaCl_2$ and $MgCl_2$ in approximately the same molar concentrations were equally suitable media for the inactivation reaction of meconium inhibitor by influenza virus A/F/51. Of the concentrations tested the optimum were M/6.3—M/10 NaCl, M/10—M/20 $CaCl_2$ and M/10 $MgCl_2$. Egg white inhibitor was destroyed by the virus equally well in NaCl and $MgCl_2$ solutions.

Homologous antiserum in dilution 1/5,000 inhibited completely the inactivation of inhibitor by Lee virus dilution 10^{-3}, and to a great extent the inactivation by A/F/51 virus in the same dilution. On the other hand, heterologous antiserum in dilution 1/50 was incapable of preventing the inactivation of inhibitor even by virus dilution 10^{-5}.

Four strains of virus A/Finland/53 isolated during an influenza epidemic in Finland in 1953 and passed through two (in one case three) egg passages, the first of which was in each case an amnion passage, were found to inactivate the meconium inhibitor somewhat better than egg white inhibitor, with the exception of one strain, which had the opposite effect when titrated against Lee indicator virus. The Lee indicator virus seemed a more »sensitive» indicator of inactivation of egg white inhibitor than the A/F/51 indicator virus, whereas both indicator viruses gave approximately similar results for meconium inhibitor. In comparison to the haemagglutination titre for chicken red cells, the enzymatic activity of the freshly isolated strains was greater than that of the older egg-adapted strain A/F/51. Thus they produced a demonstrable inactivation of inhibitor in concentrations 200 times as low as those in which they produced haemagglutination.

The significance of the inhibitor gradient phenomenon in the demonstration of small virus amounts was studied using Lee and A/F/51 viruses and meconium and egg white inhibitors. Both viruses destroyed egg white inhibitor in the same manner. The Lee virus was a considerably more »sensitive» indicator than the A/F/51 virus. For meconium inhibitor the A/F/51 virus was more active than the Lee, and greater inactivation was demonstrated in titrations with the homologous indicator virus.

The effect of red cells on the measurement of virus enzyme activity was also studied by titrations of inactivation of the meconium inhibitor against chicken cells of two different sensitivities. Using the two kinds of cells, different degrees of inactivation were demonstrated by the same indicator virus.

As the virus amounts demonstrated in the manner described were of the same order of magnitude as has been found in the throat washings of influenza patients, experiments were also carried out to demonstrate the influenza virus direct from the throat washings of influenza patients by means of red cell adsorption and elution and the enzymatic activity of the virus. A number of samples taken from patients with influenza during an influenza epidemic in Finland as well as samples from persons with respiratory infections and from healthy persons were tested.

A factor which inactivated the inhibitor was demonstrated in the throat washings not only of the patients with influenza but also of the patients with respiratory infections and of healthy subjects. A further observation made in the course of the investigation was the production into the broth by the common throat bacteria of a factor which was adsorbed onto chicken red cells and capable of inactivating egg white inhibitor. It was concluded that the presence of such a factor probably explains the egg white inhibitor-destroying activity that was observed also in the throat washings of persons with respiratory infections and healthy persons.

Although the series studied comprised only a few cases, the results obtained served to give an indication that this method appears to offer a possibility to demonstrate whether or not the inactivation is produced by the influenza virus, and if so, to determine at the same time the type of influenza virus responsible for the infection. Thus it would be possible to make a diagnosis of influenza *in vitro* within *c.* 24 hours.

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AUS DEM PHYSIOLOGISCH-CHEMISCHEN INSTITUT DER PHILIPPS-UNIVERSITÄT,
MARBURG/LAHN, DEUTSCHLAND

ÜBER DAS VORKOMMEN VON FREIEN NUCLEOTIDEN IN RUHENDEN UND WACHSENDEN GEWEBEN

VON

JUSSI J. SAUKKONEN

HELSINKI 1956

HELSINKI 1956
MERCATORIN KIRJAPAINO

INHALTSVERZEICHNIS

VORWORT

Die vorliegende Arbeit wurde in den Jahren 1954—1955 im Physiologisch-chemischen Institut der Philipps-Universität, Marburg/Lahn, durchgeführt.[1]

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[1] Ein Teil der Befunde wurde — zusammen mit Ergebnissen über den Einbau von radioaktiven Vorstufen in freie und polymerisierte Nucleotide — in einem Referat auf dem 3. Internationalen Kongress für Biochemie in Brüssel (1955) erwähnt.

VERZEICHNIS DER ABKÜRZUNGEN

A2MP	=	Adenosin-2′-monophosphat
A3MP	=	Adenosin-3′-monophosphat
AMP	=	Adenosin-5′-monophosphat
ADP	=	Adenosin-5′-diphosphat
ATP	=	Adenosin-5′-triphosphat
Ad_x	=	Adenosinphosphatderivate (*nicht* A2MP, A3MP, AMP, ADP, ATP)
G2MP	=	Guanosin-2′-monophosphat
G3MP	=	Guanosin-3′-monophosphat
GMP	=	Guanosin-5′-monophosphat
GDP	=	Guanosin-5′-diphosphat
GTP	=	Guanosin-5′-triphosphat
GDPM	=	Guanosin-5′-diphosphat-mannose
C2MP	=	Cytidin-2′-monophosphat
C3MP	=	Cytidin-3′-monophosphat
CMP	=	Cytidin-5′-monophosphat
CDP	=	Cytidin-5′-diphosphat
CTP	=	Cytidin-5′-triphosphat
U2MP	=	Uridin-2′-monophosphat
U3MP	=	Uridin-3′-monophosphat
UMP	=	Uridin-5′-monophosphat
UDP	=	Uridin-5′-diphosphat
UTP	=	Uridin-5′-triphosphat
UDPA	=	Uridin-5′-diphosphat-N-acetylglucosamin
UDPG	=	Uridin-5′-diphosphat-glucose
UDPGa	=	Uridin-5′-diphosphat-galactose
UDPGl	=	Uridin-5′-diphosphat-glucuronsäure
IMP	=	Inosin-5′-monophosphat

DPN	=	Diphosphopyridinnucleotid
TPN	=	Triphosphopyridinnucleotid
FAD	=	Flavin-adenin-dinucleotid
CoA	=	Coenzym A
HS	=	Harnsäure
RNS	=	Ribonucleinsäure
DNS	=	Desoxyribonucleinsäure
P_i	=	anorganisches Orthophosphat
PP	=	anorganisches Pyrophosphat

A EINLEITUNG UND PROBLEMSTELLUNG

In den vergangenen zwanzig Jahren hat die Biochemie eine so grosse Umwandlung erfahren, dass man ohne Übertreibung von einer Revolution in ihren Aspekten sprechen kann.

Besonders durch die Lehre von der *Funktion der Phosphorsäure* wurde die Biochemie aus der Starre der schematischen zur lebendig-dynamischen Betrachtungsweise geführt. Unsere heutigen Kenntnisse über die Bedeutung des Phosphates verdanken wir einerseits den grundlegenden Entdeckungen von Meyerhof und seiner Schule, von Lundsgaard sowie von Fiske und Subbarow bei der Erforschung der Glykolyse und andererseits den Arbeiten, die zur Formulierung des Begriffes vom »energiereichen Phosphat» bzw. der »energiereichen Bindung» durch Lipmann, Kalckar und Lynen führten. (1, 2, 3, 4).

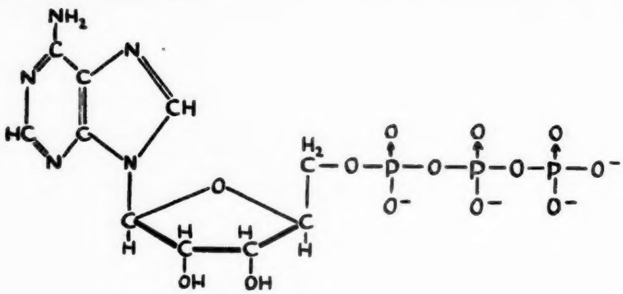
Im Zuge dieser stürmischen Entwicklung blieben Verallgemeinerungen nicht aus und führten zu Schlussfolgerungen, deren endgültige Berechtigung zum Teil noch erwiesen werden muss. Diese Entwicklungen und Überlegungen gingen im wesentlichen auf eine Schlüsselsubstanz, die 1929 gleichzeitig von Lohmann (5) und Fiske und Subbarow (82) entdeckte Adenosin-5′-triphosphorsäure zurück. Im gleichen Zeitraum wurden als frei-vorkommende Nucleotide die Mono- und Diphosphorsäureester des Adenosins — und später — die an der Wasserstoffübertragung beteiligten Transportmetaboliten Diphosphopyridinnucleotid und Triphosphopyridinnucleotid sowie Flavin-Adenin-enthaltende Verbindungen, die ebenfalls als Wasserstoffüberträger dienen, entdeckt [vgl. (6) Vol. II, (88) und (89)].

Bei der Fülle der neuen Entdeckungen fand der naheliegende Gedanke, dass auch andere Nucleotide, die nicht, wie Adenosin-5′-triphosphorsäure und deren niederphosphorylierte Verwandte ADP und AMP, Adenin als Base enthalten, in freier und auch energie-

reicher Form vorkommen können, zunächst keine Beachtung. Dies erscheint umso verwunderlicher, als das Vorkommen von Nucleotiden mit anderen Basen als Adenin in den Nucleinsäuren seit den Arbeiten von Kossel, Levene u.a. (vgl. 6) bekannt war.

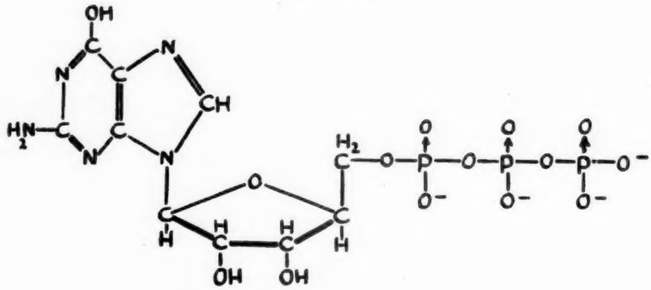
Im Jahre 1948 fand Leloir im Rahmen seiner Untersuchungen über den Kohlehydratstoffwechsel von Hefen eine weitere Gruppe von freien Nucleotiden, die als Base das Pyrimidinderivat Urazil enthalten. Weitere Arbeiten von Leloir und seinem Arbeitskreis sowie von Park erweiterten diese Befunde und führten zur Entdeckung und Isolierung von 5′-Urazil-nucleotiden und einer Reihe von Uridin-5′-diphosphat-derivaten wie UDP-N-acetylglucosamin, UDP-glucose bzw. -galactose, UDP-glucuronsäure und UDP-peptide [vgl. die zusammenfassende Darstellung von Leloir (7)].

Nachdem W. E. Cohn (49) bei seinen Untersuchungen über Struktur und Bestandteile von Nucleinsäuren *Ionenaustauscher für die Trennung von Nucleotiden* mit ausgezeichnetem Erfolg benutzte, fand diese Methodik rasch eine weite und fruchtbare Anwendung in der gesamten Biochemie. Erst kürzlich gelang es im Zusammenhang mit Untersuchungen über die Biosynthese von Nucleinsäuren in Säugetiergeweben und Tumoren durch Anwendung einer Modifikation (8) des Cohn'schen Verfahrens (49) aus den säurelöslichen Extrakten biologischen Materials neben den 5′-Mono-, Di- und Triphosphaten von Adenosin die analogen Phosphorsäureester von Uridin, Cytidin und Guanosin nachzuweisen und zu isolieren [Hurlbert und Mitarbeiter (8), Schmitz und Mitarbeiter (9, 10, 11, 12, 13)]. Bereits zuvor hatten Hurlbert (14, 15), sowie Hurlbert und Potter (16, 17) bei ihren Untersuchungen über den Einbau von C^{14}-markierter Orotsäure in die Nucleinsäuren der Rattenleber das Vorkommen von freier 5′-Uridylsäure und einiger ihrer Derivate im säurelöslichen Extrakt dieses Gewebes beobachtet. Bergkvist und Deutsch (18) berichteten ebenfalls über die Wahrscheinlichkeit des Vorhandenseins von Uridin- und Guanosin-5′-triphosphat in Extrakten aus der quergestreiften Muskulatur des Kaninchens. Ein Teil dieser Verbindungen wurde inzwischen auch in der Milchdrüse des Meerschweinchens gefunden [Smith und Mills (19)]. Weitere Untersuchungen von Schmitz und Mitarbeitern (20, 21, 22, 23) zeigten das Vorkommen der 5′-Mono-, Di- und Triphosphorsäureester von Adenosin, Guanosin, Cytidin und Uridin sowie von UDP-derivaten in verschiedenen Tumoren und normalen Geweben.



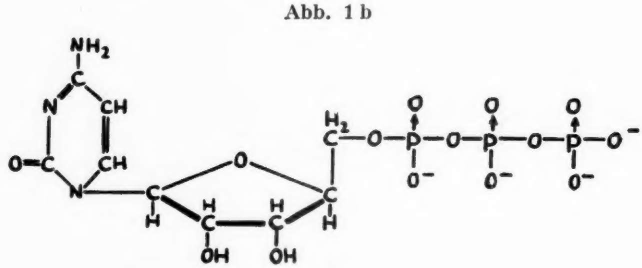
Adenosin-5'-triphosphat

Abb. 1 a



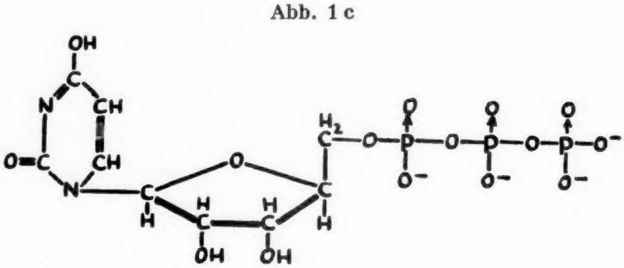
Guanosin-5'-triphosphat

Abb. 1 b



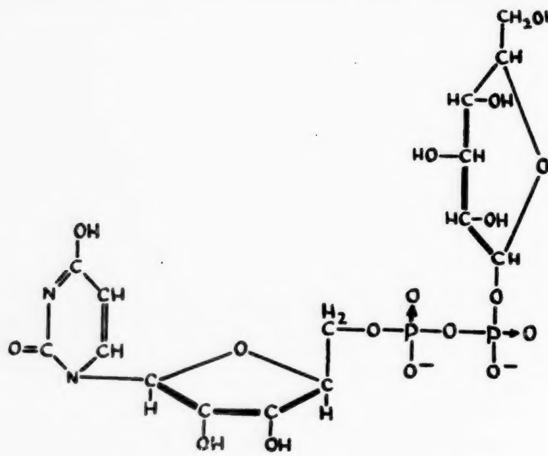
Cytidin-5'-triphosphat

Abb. 1 c



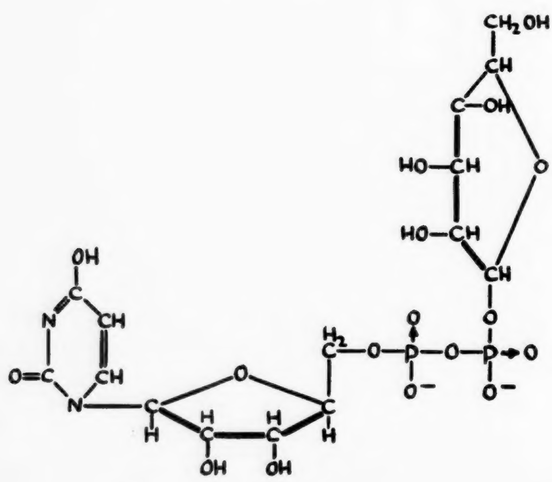
Uridin-5'-triphosphat

Abb. 1 d



Uridin-5′-diphosphat-glucose

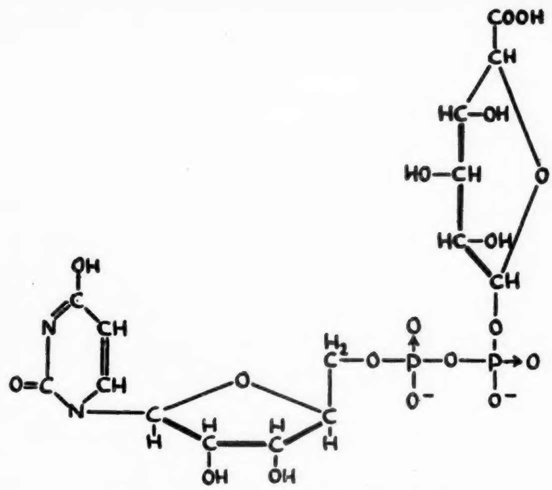
Abb. 2 a



Uridin-5′-diphosphat-galactose

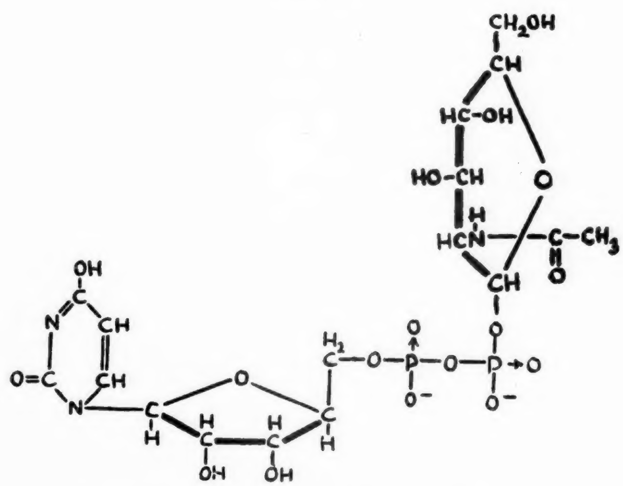
Abb. 2 b

Auf Grund der an den neuen Nucleotiden durchgeführten chemischen und enzymatischen Analysen wurden, analog den durch Synthese bestätigten Strukturen von AMP, ADP und ATP (24), für die neuen Nucleotide folgende Konstitutionsformeln erwogen (7, 20) (Abb. 1 a—d), die inzwischen von Khorana und seinen



Uridin-5′-diphosphat-glucuronsäure

Abb. 2 c



Uridin-5′-diphosphat-N-acetylglucosamin

Abb. 2 d

Mitarbeitern durch Synthese bewiesen wurden (25, 26, 27, 28). Die von Leloir (7) diskutierten Strukturen einiger Uridin-5'-diphosphatderivate (Abb. 2 a—d) bedürfen noch des endgültigen Beweises durch die Synthese.

Während über die Bedeutung der freien Adenin- und Urazil-

nucleotide als Transportmetaboliten in der Literatur eine grosse Anzahl von Befunden vorliegt, sind unsere Kenntnisse über analoge Funktionen der Guanosin- und Cytosin-nucleotide erst jüngsten Datums und daher noch unvollständig. 1954 isolierten Sanadi und Mitarbeiter (29, 30) aus Herzmuskel ein lösliches Enzymsystem, welches beim Abbau von Succinyl-CoA die Phosphorylierung von Adenosin-5'-diphosphat zu Adenosin-5'-triphosphat katalysiert.

Dieses Enzymsystem besteht aus mindestens zwei Fermenten: »Phosphorylierungsenzym» und »GTP—ADP-Transphosphorylase». Beide Fermente benötigen Guanosin-5'-diphosphat als Coferment. Sanadi's Befunde lassen sich in folgenden Gleichungen zusammenfassen:

1) Phosphorylierungsenzym:

Succinyl-CoA + GDP + $P_i \rightleftharpoons$ Bernsteinsäure + CoA + GTP

2) GTP—ADP—Transphosphorylase:

GTP + ADP $\rightleftharpoons$ GDP + ATP

Erst 1955 gelang es, eine Coenzymfunktion für die freien Cytosinnucleotide nachzuweisen. Nach den Untersuchungen von Kennedy und Weiss (31) sind Cytidinphosphorsäureester an der Lecithinsynthese wie folgt beteiligt:

1) CTP + Phosphorylcholin $\rightleftharpoons$ Cytidin-5'-diphosphat-cholin + PP.
2) Cytidin-5'-diphosphat-cholin + Akzeptor $\rightleftharpoons$ Lecithin + CMP.

Nach der Meinung der Autoren handelt es sich bei dem in Gleichung 2 teilnehmenden Akzeptor um ein α, β-Diglycerid.

In den zahlreichen Untersuchungen über die Biosynthese der Nucleinsäuren [vgl. (6)], wurde der *»pool»* der freien Nucleotide des säurelöslichen Gewebeextraktes nur wenig beachtet. Erst nachdem durch moderne Untersuchungsmethoden (Papier- bzw. Ionenaustauschchromatographie, Anwendung von durch Isotope markierten Verbindungen etc.) nachgewiesen werden konnte, dass im säurelöslichen Gewebeextrakt die gleichen Nucleotide in freier und teilweise auch energiereicher Form vorliegen, die in der Ribonucleinsäure polymerisiert vorkommen, gewann die Frage nach den *Beziehungen zwischen freien und polymerisierten Nucleotiden* an Bedeutung. Die Möglichkeit eines Abhängigkeitsverhältnisses zwischen

den verschiedenen Zustandsformen der Nucleotide, d.h., dass die freien Nucleotide neben ihren Aufgaben als Transportmetaboliten auch eine Funktion als präformierte Bausteine für die Nucleinsäuresynthese zu erfüllen haben, wurde inzwischen experimentell untersucht. Hurlbert (14, 15) sowie Hurlbert und Potter (16, 17) bestimmten an Extrakten aus Rattenlebern die spezifischen Aktivitäten der freien und polymerisierten Pyrimidinnucleotide nach der Injektion von C^{14}-markierter Orotsäure. LePage (32) sowie Edmonds und LePage (33, 34) führten ähnliche Versuche an Leber- und Tumorgeweben von Ratten, denen zuvor Glycin-2-C^{14} intraperitoneal injiziert worden war, durch. Tyner und Mitarbeiter (35, 36) bestimmten an Ratten zu verschiedenen Zeitpunkten nach intraperitonealer Injektion von durch P^{32}-markiertem anorganischen Phosphat die Radioaktivitätsgehalte des säurelöslichen Gewebeextraktes und der Nucleinsäuren. Weiterhin wurde die zeitliche Verteilung von markiertem Kohlenstoff aus Glucose-1-C^{14} [Schmitz und Mitarbeiter (10, 11, 13)] in den Pentosen und aus Glycin-2-C^{14} [Schmitz und Saukkonen (37)] in den Purinbasen der isolierten freien und polymerisierten Nucleotide an tumortragenden Tieren untersucht. Diese Autoren fanden, dass der jeweils verwendete radioaktive Vorläufer zunächst in die Nucleotide des säurelöslichen Extraktes und erst dann — unter gleichzeitiger Abnahme der Radioaktivität in diesen Verbindungen — in die Nucleinsäuren eingebaut wurde. Diese Ergebnisse unterstützten die Annahme, dass *die freien Nucleotide* — neben ihren Funktionen als Transportmetaboliten — *als Vorläufer für die Nucleinsäuren in Betracht kommen können* [vgl. Potter (90) und Hurlbert (94)].

Bildlich gesprochen ist durch die Entdeckung der neuen Nucleotide ein Tor aufgestossen worden und die Beschreitung des dahinterliegenden Weges lässt eine Bereicherung unserer Kenntnisse über die Vorgänge in der lebenden Zelle erhoffen. Gleichzeitig ergeben sich aber auch neue Fragen und Probleme. Es ist der Zweck dieser Arbeit, durch eine vergleichende Untersuchung über den Bestand ruhender und wachsender Gewebe an freien Nucleotiden zu einer Erweiterung unserer Kenntnisse über die chemische Zusammensetzung der Zelle beizutragen. Die Untersuchungen wurden durchgeführt unter besonderer Berücksichtigung der Frage:

Bestehen zwischen ruhenden und wachsenden Geweben Unterschiede in der Zusammensetzung ihrer Reservoirs an freien Nucleotiden?

B EXPERIMENTELLER TEIL

I METHODEN

1) PRINZIP DER METHODE

Das zu untersuchende Gewebe wird mit Perchlorsäure [Neuberg und Mitarbeiter (79)] enteiweisst, homogenisiert und extrahiert. Nach Ausfällung des Perchloratanions durch Zusatz von Kalilauge wird der neutrale Extrakt in einer modifizierten Form [Hurlbert und Mitarbeiter (8), Schmitz und Mitarbeiter (9)] nach Cohn (49) durch Anionenaustauschchromatographie aufgetrennt. Die Eluierung erfolgt durch langsam ansteigende Konzentration von Ameisensäure, Ammonformiat, bzw. durch ein Gemisch beider. Die einzelnen Fraktionen werden auf ihre Absorption von ultraviolettem Licht, ihre Gehalte an Phosphorsäure, Pentose und falls erforderlich, an anderen Verbindungen wie Hexosen, deren Derivate und Aminosäuren untersucht (8, 9).

2) HERSTELLUNG DER SÄURELÖSLICHEN EXTRAKTE

a) Säugetiergewebe und feste Tumoren

Die Gewebe der durch Dekapitierung getöteten Tiere [1] wurden so schnell wie möglich herausgeschnitten, in flüssiger Luft gefroren, im Stahlmörser pulverisiert (38) und in einem Potter-Elvehjem-Homogenisator (39) zerkleinert. Um einen Abbau von labilen Verbindungen, wie z.B. ATP, weitmöglichst auszuschliessen, wurde die Extraktion bei 0— +2° durchgeführt. Für die Extraktion wurden

[1] Für die Untersuchungen wurden ca. 200 g schwere Ratten bzw. 20—25 g schwere Mäuse der Zucht von E. Balweg, Sinsheim (Baden) und der Zucht des Pharmakologischen Instituts der Philipps-Universität, Marburg/L. benutzt.

zwei Volumina 0.6 n Perchlorsäure[1] verwendet, so dass die Endkonzentration der Säure etwa 0.4 n betrug (8). Der säureunlösliche Rückstand wurde in der Kälte abzentrifugiert (20 min, 15000 $\times$ g, 0— +2°), nochmals mit einem Volumen 0.2 n Perchlorsäure (8) extrahiert und unter gleichen Bedingungen zentrifugiert. Die beiden Überstände wurden vereinigt und durch Hinzugabe von Kalilauge neutralisiert (Phenolrot als Indikator). Vor Abzentrifugierung des ausgefallenen Kaliumperchlorats wurde der säurelösliche Extrakt in ein Aceton-Eis-Bad gestellt, um ein möglichst quantitatives Ausfallen des Salzes zu erreichen.

b) Ascitestumoren

Die untersuchten Ascitestumoren wurden zwischen dem sechsten bis achten Tage nach der Überimpfung durch Punktion des Peritoneums gewonnen und die Zellen durch Zentrifugieren vom Serum abgetrennt (20 min, 15000 $\times$ g, 0— +2°). Die Zellen wurden dann ohne Behandlung mit flüssiger Luft wie feste Gewebe durch Homogenisieren und Zentrifugieren in 0.6 n Perchlorsäure extrahiert und aufgearbeitet.

Die Extraktion der Ascitessera wurde durch Zusatz von 2 Volumina 0.6 n Perchlorsäure und Umrühren (0— +2°, 10 min) bewirkt und ihre Aufarbeitung bis zur Chromatographie erfolgte analog den festen Geweben.

c) Hefezellen

Für die Untersuchungen über den Gehalt von Hefen an freien Nucleotiden wurden sogenannte Bäcker- und Bierhefen verwendet. Die in dieser Arbeit mitgeteilten Ergebnisse über den Nucleotidgehalt von Hefezellen beziehen sich besonders auf eine Bäckerhefe der Firma Dr. Fritz Hillringhaus, Getreide-Presshefefabrik und Brennerei, Wuppertal-Barmen. Durch Vermittlung der Farbenfabriken Bayer, A. G., Wuppertal-Elberfeld, wurde ein Trockenpräparat des säurelöslichen Extraktes aus dieser Hefe wie folgt hergestellt: 300 g der auf Melasse-Nährboden gezogenen obergärigen Hefe wurden zu dem Zeitpunkt des maximalen Wachstums dem Tank entnommen, sofort in Eis gekühlt und etwa 15 min später

[1] Falls nicht anders vermerkt, wurden für die Arbeit nur »pro analysi«-Chemikalien der Fa. E. Merck, Darmstadt, oder gleichwertige Präparate der Fa. Farbenfabriken Bayer A. G., Wuppertal-Elberfeld, verwendet.

unter den für Säugetiergewebe und Tumoren angegebenen Bedingungen extrahiert. Der neutralisierte säurelösliche Extrakt wurde der Gefriertrocknung unterworfen; die Ausbeute betrug 10 g Trockensubstanz. Für die verschiedenen chromatographischen Analysen wurde eine abgewogene Menge in 5—10 Volumina aqua bidest. aufgenommen, das p_H auf den Neutralpunkt eingestellt und dann an den Austauscher adsorbiert.

Unter gleichen Bedingungen wurden säurelösliche Extrakte aus Löwenhefe (München), Sandvosshefe (München) und Bopp-Beyerhefe (Marburg/Lahn) hergestellt.

3) EXTRAKTION DER GEMISCHTEN NUCLEINSÄUREN

Die Nucleinsäuren wurden nach der von Hurlbert und Potter (16) angegebenen Vorschrift aus dem säureunlöslichen Rückstand mit 10%igem NaCl extrahiert und mit zwei Volumina Äthylalkohol[1] (95%) gefällt. Die Extraktionsbedingungen waren wie folgt:

a) Nach zweimaliger saurer Extraktion (s. oben) wurde der Rückstand nochmals mit 2 Volumina 0.2 n Perchlorsäure gewaschen und 30 min zentrifugiert (15000 × g, 0— +2°). Rückstand in zweieinhalb Volumina 95%igem Äthanol und 0.6 n Perchlorsäure (5 : 1, v. : v.) aufgenommen und zentrifugiert.

b) Rückstand in 5 Volumina 95%igem Äthanol aufgenommen und zentrifugiert; Überstand verworfen. Diese Alkoholextraktion wurde zweimal durchgeführt.

c) Rückstand in Äthanol/Äthyläther (3 : 1, v. : v.) aufgenommen, auf dem Wasserbad bei 35—40° unter Umrühren für 30 min extrahiert und bei Zimmertemperatur zentrifugiert (20°, 40 min, 5000 × g); Überstand verworfen.

d) Rückstand auf 0° abgekühlt und mit zwei Volumina 10%iger NaCl-Lösung versetzt, unter häufigem Umrühren bei 0— +2° für ein bis zwei Stunden belassen und dann zentrifugiert; Überstand verworfen.

e) Rückstand mit zweieinhalb Volumina 10%iger NaCl-Lösung versetzt, im Eisbad auf p_H 7 eingestellt und für 40 min gekocht. Nach 10 min wurde die Erhitzung unterbrochen, die Lösung im Eisbad abgekühlt, das p_H kontrolliert und falls notwendig, erneut auf 7 eingestellt, da nur bei genauer Beachtung des p_H-Wertes die

[1] Mit Methanol vergällt.

Ribo- und Desoxyribonucleinsäuren in hochpolymerer Form erhalten bleiben (die ersteren werden im alkalischen und die letzteren im sauren Milieu depolymerisiert). Nach Beendigung der Extraktion wurde die Lösung auf ~ +2° abgekühlt, zentrifugiert und der Rückstand nochmals unter gleichen Bedingungen extrahiert, die vereinigten Überstände nach Filtrierung durch Glaswolle mit zweieinhalb Volumina eiskalten Äthanols (95%) versetzt und über Nacht im Kaltraum (0— +2°) belassen.

f) Die ausgefallenen gemischten Nucleinsäuren wurden abzentrifugiert, dreimal mit 95%igem Äthanol gewaschen und im Exsiccator über $CaCl_2$ getrocknet.

4) DARSTELLUNG VON MONONUCLEOTIDEN AUS NUCLEINSÄUREN

Die Ribonucleinsäuren wurden durch Inkubierung des gemischten Na-Nucleats während 20 Stunden bei 38° mit 0.1 n NaOH (1.0 ml pro mg getrocknetes gemischtes Na-Nucleat) depolymerisiert (8). Die unter diesen Bedingungen nicht depolymerisierten Desoxyribonucleinsäuren wurden durch Ansäuern der Lösung im Eisbad mit Salzsäure (p_H1) und Zusatz von zweieinhalb Volumina Äthanol (95%) präzipitiert und durch Zentrifugierung abgetrennt. Der Überstand (Ribonucleotide) wurde neutralisiert, mit vier Volumina Äthanol (95%) versetzt und nochmals wie oben angesäuert. Diese Alkohol-Säurebehandlung in der Kälte wurde dreimal durchgeführt, um DNS-freie Ribonucleotide zu erhalten. Nach der letzten Alkohol-Säurebehandlung wurde der Überstand neutralisiert und zur Analyse auf Nucleosid-2′ — und — 3′-phosphorsäureester verwendet (Chromatographie wie unter »Methoden», S. 20).

Die Depolymerisation der Desoxyribonucleinsäuren zu den entsprechenden Oligonucleotiden wurde mittels Desoxyribonuclease[1] (40) durchgeführt und der Abbau dieser zu den Mononucleotiden mit Phosphodiesterase aus *Crotalus Adamanteus*[2] erreicht (41, 42). Zur Vermeidung einer Verunreinigung der DNS durch RNS erfolgte vor Beginn des enzymatischen Abbaues eine 2—3-malige Wiederaufnahme der DNS in 3 Volumina 0.1 n NaOH mit anschliessender Erhitzung auf 80° für die Dauer von 20 min; die DNS wurde dann wieder unter den oben angegebenen Bedingungen ausgefällt.

[1] Handelsübliches Präparat der Fa. General Biochemicals, Inc., Chagrin Falls, Ohio, USA.

[2] Handelsübliches Trockenpräparat der Fa. Ross Allen's Reptile Institute, Silver Springs, Florida, USA.

5) HERSTELLUNG DER IONENAUSTAUSCHSÄULEN

Für die Darstellung der im säurelöslichen Extrakt enthaltenen bzw. durch alkalische oder enzymatische Depolymerisation der Nucleinsäuren gewonnenen Mononucleotide wurden die stark basischen Austauscher Dowex-1 (2% und 8% Cross-linkage) und Dowex-2 (10% Cross-linkage)[1] verwendet. Die in der Chloridform gelieferten Austauscher wurden durch Waschen mit 4 n HCOONa in die Formiatform übergeführt (8). Um den Austauscher von bei der UV-Analyse möglicherweise störenden Stoffen zu befreien, wurde dieser nach Umwandlung in die Formiatform mit 3—5 Volumina ca. 23 n HCOOH beschickt und anschliessend mit bidest. Wasser neutral gewaschen.

Als Kationenaustauscher wurde Dowex-50, H^+ (8% Cross-linkage)[1], das zuvor mit 6 n HCl gereinigt war, verwendet.

Das Austauscherbett, dessen jeweils verwendete Grösse bei den einzelnen Chromatogrammen angegeben ist, wurde in Sektionen von je ein bis zwei cm Höhe gepackt, um die Eluierung der Verbindungen in Form scharfer UV-Absorptionsmaxima zu begünstigen.

6) ELUIERUNG DER IONENAUSTAUSCHER UND AUSWERTUNG DER CHROMATOGRAMME

Die Eluierung wurde mit Hilfe der in Abb. 3 angegebenen Vorrichtung durchgeführt. Die in dem Kolben A (=Reservoirflasche) enthaltene Säure oder Pufferlösung wurde mittels Pressluft durch einen Polyäthylenschlauch in den Kolben B, der zu Beginn des Versuches eine bestimmte Menge Wasser enthielt, hinübergedrückt. Die in dem Kolben B (= Mischflasche) langsam ansteigende Konzentration der Eluierungsflüssigkeit wirkt dann auf die Säule. Die aus dem Kolben A in die Mischflasche zufliessende Säure wird mit Hilfe eines magnetischen Rührwerkes vermischt. Der Anstieg der auf den Austauscher wirkenden Säurekonzentration kann nach folgender Formel (8) berechnet werden:

Konzentration der Eluierungsflüssigkeit =

$$\begin{array}{l}\text{Konzentration der Eluierungsflüssigkeit}\\ \text{in der Reservoirflasche}\end{array} \cdot \left[\frac{\left(\text{Antilog}\ \frac{X}{2.3\ V}\right) - 1}{\left(\text{Antilog}\ \frac{X}{2.3\ V}\right)}\right]$$

$$\text{wobei}\ \frac{X}{V} = \frac{\text{Menge an verbrauchter Eluierungsflüssigkeit}}{\text{Volumen der Mischflasche}}$$

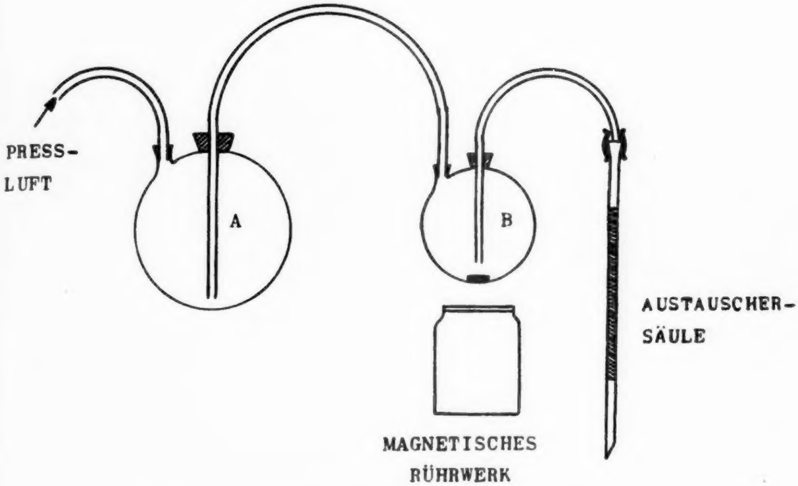


Abb. 3. — Vorrichtung zur kontinuierlichen Eluierung

Unter Berücksichtigung der Grösse des Austauscherbettes kann der Anstieg der Säurekonzentration durch Variation des Volumens der Mischflasche kontrolliert werden.

Die Auftrennung des Eluates in Fraktionen von 4 bis 5 ml wurde an einem automatischen Fraktionssammler durchgeführt[1].

Alle Fraktionen eines Chromatogramms wurden zunächst in ihrem Lösungsmittel in einem Beckman-Spektrophotometer (Modell DU) gegen Wasser bei 260 und 275 mμ gemessen. Der Quotient E_{275}/E_{260} dient als praktisch brauchbarer Anhaltspunkt für die weitere Identifizierung der Fraktionen, da er für die einzelnen Nucleotide charakteristisch ist. Unter diesen Bedingungen betragen die E_{275}/E_{260}-Quotienten für die Adenin-, Guanin-, Urazil- bzw. Cytosin-enthaltenden Nucleotide ~ 0.4; ~ 0.7; ~ 0.6; ~ 1.3—1.7. Diese Werte sind gewissen Schwankungen unterworfen, die vor allem von folgenden Faktoren abhängig sind: 1.) Mehr oder weniger unvollständige Auftrennung der einzelnen aufeinanderfolgenden Verbindungen; 2.) Einfluss des p_H-Wertes; 3.) Konzentration der im UV absorbierenden Eluierungsmittel und — besonders bei

zu Seite 20:
[1] Vernetzungsgrad nach Angaben der Hersteller: Dow Chemical Company, Midland, Michigan, USA.

zu Seite 21:
[1] »Volumetric Fractionator« der Fa. Gilson, Medical Electronics, Madison, Wisc., USA.

kleinen »*peaks*« — das Verhältnis der Extinktionen von Elutierungsflüssigkeit und zu messender Fraktion. Die E_{275}/E_{260}-Quotienten der reinen Substanzen können aus Tab. 2 errechnet werden.

Da durch die in der Reservoirflasche zu Beginn der Eluierung enthaltene 1 oder 4 n Ameisensäure nur ein Teil der im Gewebe vorhandenen freien Nucleotide eluiert wird (bei Verwendung von 4 n HCOOH bis zur ADP einschliesslich, vgl. Abb. 6), wurde die Gewinnung der restlichen Verbindungen durch Zusatz von geringen Mengen an Ammoniumformiat bewirkt. Die jeweils verwendeten Konzentrationen an Säure, Puffer bzw. eines Säure-Puffergemisches, die Grösse der Mischflasche und die Zeitpunkte, zu denen die Eluierungsflüssigkeit gewechselt wurde, sind aus den Legenden der einzelnen Abbildungen zu entnehmen[1].

Im Laufe der Untersuchungen erwies es sich als vorteilhaft in den Fällen, in denen die Eluierung mit 1 n HCOOH in der Reservoirflasche begonnen wurde, diese nicht wie bisher mit 4 n, sondern mit 3 n Ameisensäure fortzusetzen. Die dadurch bedingte zeitliche Ausdehnung der Chromatographie ergibt aber in geeigneten Fällen eine wesentlich bessere Auftrennung von Gewebeextrakten.

Verschiedene Fraktionen eines solchen Chromatogrammes (im folgenden auch als S—1 abgekürzt) wurden nach Messung der Absorption bei 260 und 275 mμ unter Berücksichtigung ihrer E_{275}/E_{260}-Quotienten vereinigt und mittels Gefriertrocknung vom Lösungsmittel befreit. Gelegentlich wurden Fraktionen, die keine Verbindungen mit labiler Phosphatbindung enthielten, im Exsiccator unter Erwärmung durch Infrarotlicht getrocknet.

Wie aus den Beschriftungen der S—1-Chromatogramme darstellenden Abbildungen ersichtlich ist, handelt es sich in den meisten Fällen um mehr oder weniger heterogene UV-Absorptionsmaxima. Eine Auftrennung dieser Maxima[2] wurde durch Rechromatographie (im folgenden auch als S—2 abgekürzt) entsprechender Sektionen an einem basischen Ionenaustauscher erreicht. Die Eluierung erfolgte durch langsam ansteigende Konzentration von Ammoniumformiatpuffer (p_H 5 ⟶ 4). Da die Nucleotide — relativ zueinander — eine unterschiedliche Ladung in Säure bzw. in Pufferlösung

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[1] Alle in dieser Arbeit beschriebenen Chromatographieen wurden bei Zimmertemperatur durchgeführt.

[2] Vor Beginn der Rechromatographie wurde jeweils eine kleine Probe des in Lösung gebrachten Gefriertrocknungsprodukts für Übersichtsanalysen (Phosphat, Ribose, etc.) abgezweigt.

aufweisen, ergibt sich daraus eine gute Auftrennung, vergleichbar der Wirksamkeit zweidimensionaler Papierchromatographie. Dafür seien folgende Beispiele gegeben:

a) Die 5'-Phosphorsäureester der Nucleoside, wie sie durch enzymatische Hydrolyse aus Ribonucleinsäure erhalten werden, erscheinen an S—1 (Abb. 4a) in der Reihenfolge CMP, AMP, GMP,

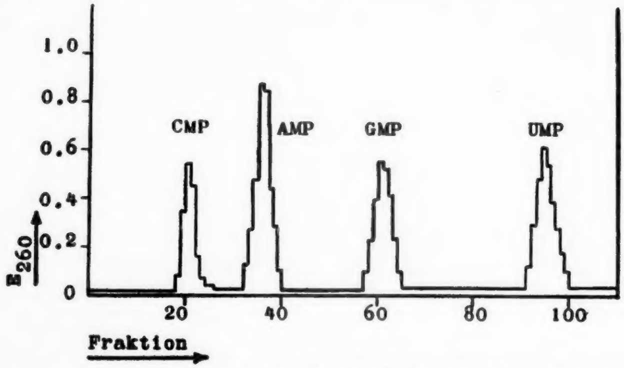


Abb. 4 a. — Reihenfolge der Nucleosid-5'-phosphorsäureester an S-1

Dowex—2, $HCOO^-$, 1.0 × 22.0 cm
Mischflasche: 500 ml H_2O
Reservoirflasche: 1-Ende 4n HCOOH, 4 ml/Fraktion

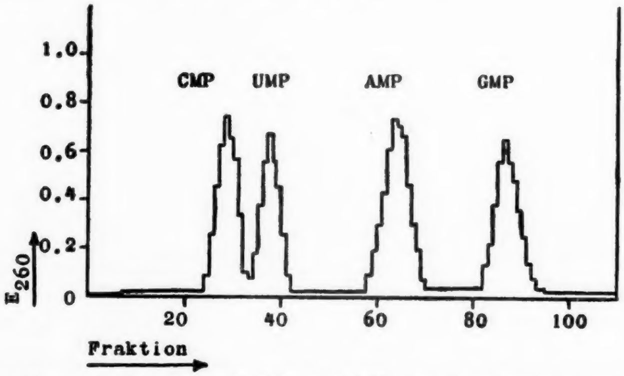


Abb. 4 b. — Reihenfolge der Nucleosid-5'-phosphorsäureester an S-2

Dowex—2, $HCOO^-$, 1.0 × 22.0 cm
Mischflasche: 500 ml H_2O
Reservoirflasche: 1-Ende 1n $HCOONH_4$, 4 ml/Fraktion

UMP, während bei der Rechromatographie an S—2 (Abb. 4 b) erst die Pyrimidinnucleotide CMP und UMP eluiert werden, denen dann die Purinnucleotide AMP und GMP folgen.

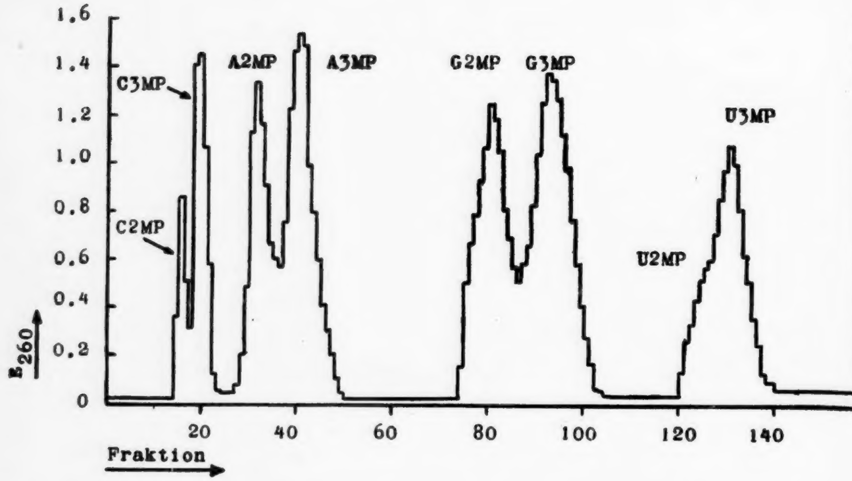


Abb. 5 a. — Reihenfolge der Nucleosid-2′- und 3′-phosphorsäureester an S-1

Dowex—2, $HCOO^-$, 1.0 × 15.0 cm
Mischflasche: 250 ml H_2O
Reservoirflasche: 1—45 1n HCOOH
46—Ende 3n HCOOH, 3 ml/Fraktion

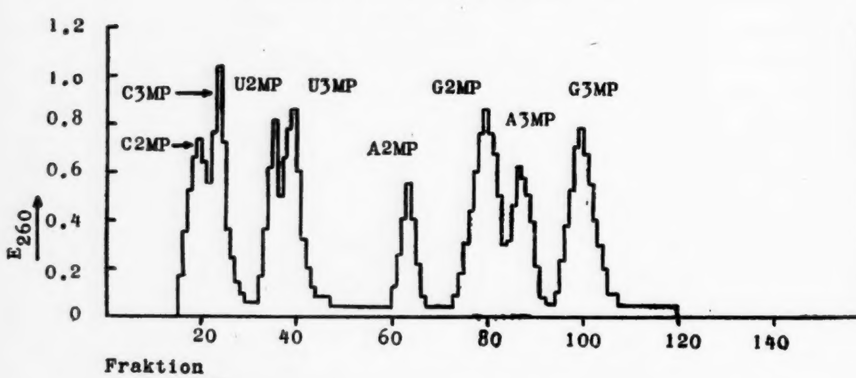


Abb. 5 b. — Reihenfolge der Nucleosid-2′- und 3′-phosphorsäureester an S-2

Dowex—2, $HCOO^-$, 1.0 × 18.0 cm
Mischflasche: 485 ml H_2O
Reservoirflasche: 1—Ende 1n $HCOONH_4$, 3.5 ml/Fraktion

b) Die durch alkalische Hydrolyse aus Ribonucleinsäure erhaltenen 2′-, 3′-Nucleotide erscheinen bei der Chromatographie an S—1 wie unter a) beschrieben, während sie bei der Rechromatographie folgende Reihenfolge einhalten:

C2MP, C3MP, U2MP, U3MP, A2MP, G2MP, A3MP, G3MP (Abb. 5 a und b).

7) IDENTIFIZIERUNG DER VERBINDUNGEN

a) Spektrophotometrische Auswertung

Die bei der Rechromatographie an S—2 erhaltenen Fraktionen wurden unter Berücksichtigung ihrer spektrophotometrischen Einheitlichkeit (E_{275}/E_{260}-Quotient) vereinigt und das Ammoniumformiat durch Gefriertrocknung entfernt. Der nicht-flüchtige Rückstand wurde in 0.001 bis 0.1nHCl aufgenommen und spektrophotometrisch untersucht. Diese Messungen erstreckten sich vor allem auf den Bereich des ultravioletten Lichtes (215—320 mμ in Säure und 220—320 mμ in Alkali); als Blindwert diente das jeweilige Lösungsmittel. Ein Teil der Absorptionsspektra wurde an einem automatisch registrierenden Spektralphotometer (Beckman, Modell DK-1) aufgenommen.

Die durch die Hydrolyse in 1 n Salzsäure (Purinnucleotide) bzw. in 11.5 n Perchlorsäure (Pyrimidinnucleotide) (43, 44) erhaltenen freien Basen wurden an kleinen Kationenaustauschsäulen (Dowex-50, H^+) nach der von LePage (32) angegebenen Methode abgetrennt und spektrophotometrisch identifiziert.

b) Pentosebestimmung

Die Pentosen wurden mit der Orzin-(Ribose) bzw. Diphenylamin-Reaktion (Desoxyribose) bestimmt (8). In einzelnen Fällen, bei denen durch vorsichtige Hydrolyse mit verdünnter Salzsäure (1 n, 30 min) Ribosephosphate von Purinnucleotiden erhalten wurden, erfolgte ihre chromatographische Auftrennung in 2′- bzw. 3′-Ester an Dowex-1 in der Monochloracetat-Form nach Groth und Mitarbeitern (45); quantitative Auswertung mittels Phosphat- und Ribosebestimmungen.

c) Phosphatbestimmung

Die Bestimmung des gesamten, des anorganischen und des labilen Phosphates erfolgte zum Teil in einer modifizierten Form (8) nach Fiske und Subbarow (46) und zum Teil nach Lowry und Mit-

arbeitern (47) oder Rockstein und Herron (48). Die Methoden zur Bestimmung von Phosphat nach Lowry und Mitarbeitern sowie nach Rockstein und Herron wurden durch anteilmässige Verringerung der Volumina der für die Analysen notwendigen Lösungen so modifiziert, dass für die Einzelbestimmungen nur kleinere Proben erforderlich sind (71). Die Phosphatbestimmungen wurden in Pyrexkolben bzw. -Gläsern durchgeführt, um einen möglichen Einfluss von Silikaten weitgehendst auszuschalten.

Die Stellung der Phosphatgruppen der Nucleosidester (2′, 3′ oder 5′) wurde in Anlehnung an die Arbeiten von Cohn (49, 50) gemäss ihrem Verhalten bei der Anionenaustauschchromatographie und der Freisetzung von anorganischem Phosphat nach saurer Hydrolyse bestimmt.

Die Charakterisierung von Nucleotiden als Nucleosid-5′-phosphorsäureester basiert neben den im vorhergehenden Absatz erwähnten Analysen auf der Messung des freigesetzten anorganischen Phosphates durch Einwirkung von 5′-Nucleotidase aus dem Schlangengift von *Crotalus Adamanteus* in modifizierter Form (9) nach Heppel und Hilmoe (51).

d) *Papierchromatographische Analysen*

Das durch Hydrolyse (0.01 nHCl, 100°, 5 min) (17) aus Uridin-5′-diphosphat-N- acetylglucosamin abgespaltene N-Acetylglucosamin wurde papierchromatographisch [Collidin-Wasser (52) und wassergesättigtes Phenol (7)] abgetrennt und sein R_f-Wert mit dem von bekanntem N-Acetylglucosamin verglichen; ausserdem wurde das Hydrolyseprodukt nach Morgan und Elson (53) quantitativ bestimmt. Glucose, Galactose und Mannose wurden ebenfalls nach Hydrolyse mit 0.01 nHCl papierchromatographisch [wassergesättigtes Phenol (83) und Butanol-Eisessig (84)] abgetrennt und quantitativ mit der Anthron-bzw. Carbazolmethode ausgewertet (54, 66). Die Glucuronsäurebestimmung erfolgte nach Dische (55, vgl. 17).

8) HERSTELLUNG DER TUMOREN

a) Die untersuchten festen Tumoren wurden durch Transplantation aus Tumortieren, die zum Teil vom Institut für experimentelle

Krebsforschung der Universität Heidelberg und zum Teil von den wissenschaftlichen Laboratorien der Farbenfabr. Bayer, A.G., zur Verfügung gestellt wurden, fortgezüchtet. Für die subkutane Transplantation von Flexner-Jobling-Carcinom, Walker-256-Carcino-Sarkom, Jensen-Sarkom und Yoshida-Sarkom wurde als Ausgangsmaterial der Tumor im Alter von 9—11 Tagen verwendet. Die Übertragung erfolgte durch 4—5 subkutane Implantate pro Tier.

b) Die verwendeten Ascitestumoren stammten aus den gleichen Instituten. Die Fortzüchtung der Tumoren erfolgte durch intraperitoneale Injektion von 0.2 ml unverdünnter Ascitesflüssigkeit eines Tieres, dessen Tumor 9—12 Tage alt war.

II. ERGEBNISSE

In diesem Abschnitt werden die Ergebnisse der durch Anionenaustauschchromatographie erzielten Auftrennungen von säurelöslichen Gewebeextrakten und depolymerisierten Nucleinsäuren aus Tumoren, Hefen und normalen Säugetiergeweben beschrieben; anschliessend wird eine tabellarische Zusammenstellung der Analysen gegeben.

1) FESTE TUMOREN

a) Yoshida-Sarkom

α) Säurelöslicher Extrakt

Die Abbildung 6 zeigt ein S—1-Chromatogramm des säurelöslichen Extraktes aus den Zellen dieses Tumors. Es handelt sich dabei um die vereinigten Tumoren von sieben Ratten am fünften Tage nach der Transplantation (8 g Feuchtgewicht). In dieser Abbildung sind wiederum die optische Dichte der aufeinanderfolgenden Fraktionen bei 260 mμ sowie der Quotient E_{275}/E_{260} aufgetragen. Nähere Einzelheiten über die Eluierungsbedingungen sind in der Legende zu dieser Abbildung enthalten.

Wie aus den Bezeichnungen der einzelnen UV-Absorptionsmaxima ersichtlich ist, enthält dieser Tumor neben den lange bekannten Adeninnucleotiden AMP, ADP und ATP auch die 5'-Mono-, Di- und Triphosphorsäureester von Guanosin, Cytidin und Uridin sowie verschiedene Uridin-5'-diphosphat-derivate wie UDP-glucose,

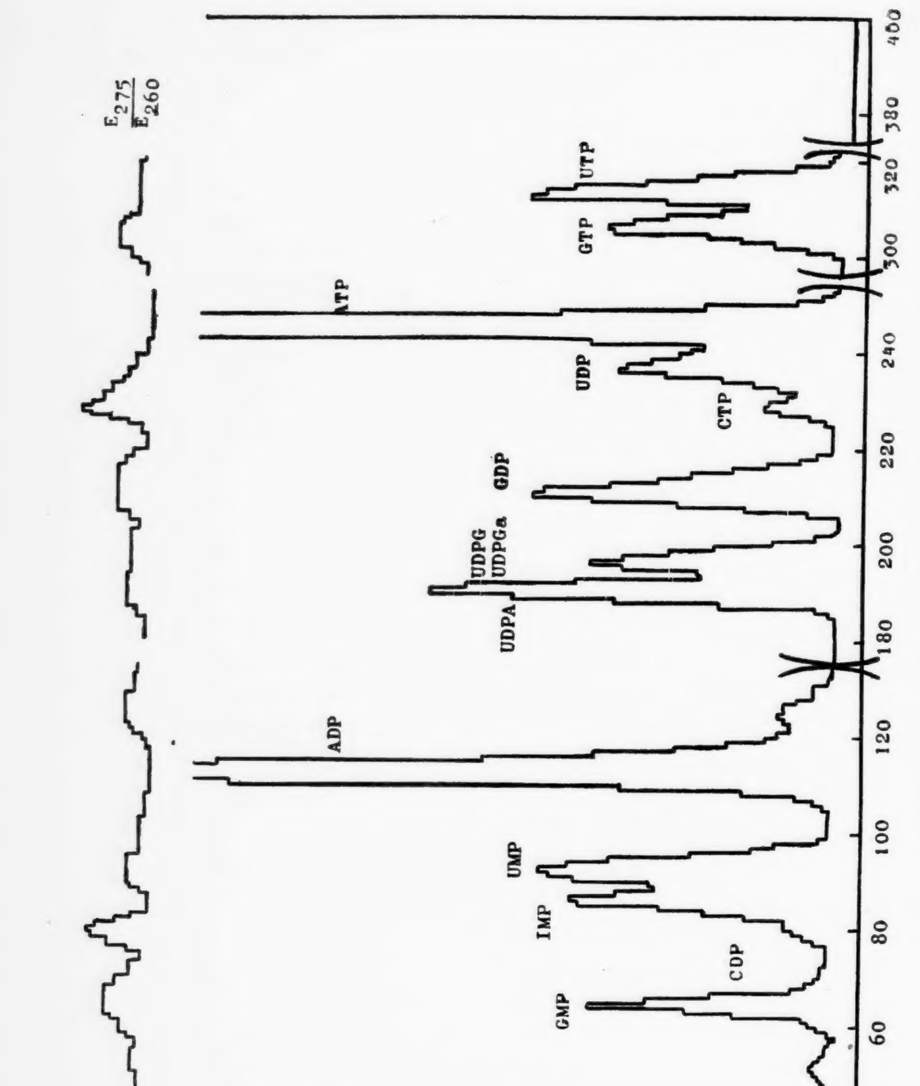


Abb. 6
Dowex—2, $HCOO^-$, 0.9×20.0 cm
Mischflasche 475 ml H_2O
Reservoirflasche: 1—140 3 n HCOOH
141—250 4 n » +0.4 n $HCOONH_4$
251—Ende 4 n » +0.8 n $HCOONH_4$, 4.2 ml/Frakt.

UDP-galactose, UDP-glucuronsäure und UDP-N-acetylglucosamin. Die Beschriftung der Absorptionsmaxima zeigt weiterhin, dass nur einem Teil von ihnen spektrophotometrische Einheitlichkeit zukommt, d.h., dass in einigen dieser Maxima mehrere Verbindungen enthalten sind. In den Fällen, in denen zwei Verbindungen dicht aufeinanderfolgen, lassen auch die Quotienten E_{275}/E_{260} erkennen, dass sich die Anfangs- bzw. Endfraktionen dieser Substanzen überschneiden. Die Ergebnisse der zur Reindarstellung der Nucleotide durchgeführten Rechromatogramme von einzelnen Sektionen des S—1-Chromatogramms (Abb. 6) sind in den Abbildungen 7—16 wiedergegeben.

Abbildung 7 stellt die Rechromatographie der Fraktionen 1—27 des S—1-Chromatogramms dar. Neben einer besseren Auftrennung der einzelnen Verbindungen voneinander fällt vor allem die Eluierungsfolge DPN, CMP, welche an S—1 umgekehrt ist, auf. Die im ersten Chromatogramm (S—1) nur auf Grund ihres sehr hohen E_{275}/E_{260}-Quotienten zu vermutende Harnsäure ist hier frei von Verunreinigungen erhalten worden. Die Fraktionen 3—6 und 11—16 enthalten Verbindungen, deren Indentifizierung noch aussteht.

Eine bessere Auftrennung der ersten Fraktionen des S—1-Chromatogramms (etwa bis zu AMP einschliesslich) kann auch dadurch erreicht werden, dass die Eluierung nicht mit 4 n HCOOH, wie im Fall des in Abbildung 6 dargestellten Chromatogramms, sondern mit 0.5 n HCOOH in der Reservoirflasche begonnen und nach Erscheinen der AMP mit 3 oder 4 n Säure fortgesetzt wird; ausserdem erwies es sich in geeigneten Fällen als günstig, die Eluierung mit 1—3 n HCOOH zu beginnen und bis zu ADP fortzusetzen. Eine derartige Eluierung ist für die Auftrennung von verschiedenen säurelöslichen Extrakten aus Tumoren und Hefen (vgl. Abb. 19 und 23) durchgeführt worden. Eine Verringerung der Volumina der ersten Fraktionen des S—1-Chromatogramms kann für die Auftrennung von Extrakten mit niederem DPN-Gehalt ebenfalls vorteilhaft versucht werden.

Das nächste Rechromatogramm (Abb. 8) umfasst die Fraktionen 61—70 des S—1-Chromatogramms, die dort mit GMP bezeichnet sind. Der grösste Teil der UV-Absorption entspricht auch hier der 5′-Guanylsäure (Fraktionen 73—83). Ausserdem finden sich aber noch zwei kleine UV-Absorptionsmaxima, über deren Identität

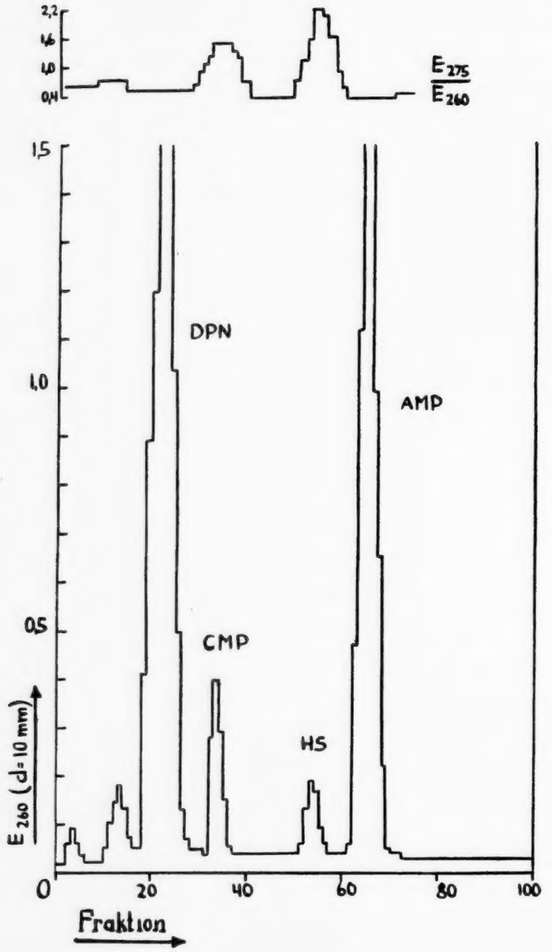


Abb. 7
Dowex—2, $HCOO^-$, 1.0×21.0 cm
Mischflasche: 485 ml H_2O
Reservoirflasche: 1—Ende 1 n $HCOONH_4$, 4 ml/Fraktion

noch keine endgültigen Aussagen gemacht werden können, da es bisher schwierig war, aus diesem Gewebe genügend Material für eine einwandfreie Analyse ihrer Spektra, ihrer Ribose- und Phosphatgehalte zu isolieren. Möglicherweise enthalten die Fraktionen 33—41 das Coenzym II (Triphosphopyridinnucleotid). In unter gleichen Bedingungen hergestellten Rechromatogrammen vergleichbarer S—1-Sektionen aus Leber (Abb. 27), die relativ reich an diesem

Coenzym ist, erscheint TPN an der gleichen Stelle (vgl. Abb. 28). Die Spektrophotometrische Auswertung der Fraktionen 23—28 lässt ebenfalls auf eine Adenin-enthaltende Verbindung schliessen (Maximum: 258 mμ, Quotient E_{275}/E_{260} : 0.5).

Die Rechromatographie der Fraktionen 76—100 von S—1 ist in Abbildung 9 dargestellt. Durch teilweise Hydrolyse des Cytidindiphosphats ist ein Teil als Monophosphorsäureester wiedergewonnen worden. Die in Abbildung 6 sehr dicht aufeinanderfolgenden Nucleotide Cytidin-5′-diphosphat, Inosin-5′-monophosphat und Uridin-5′-monophosphat sind hier gut voneinander getrennt worden. Ausser-

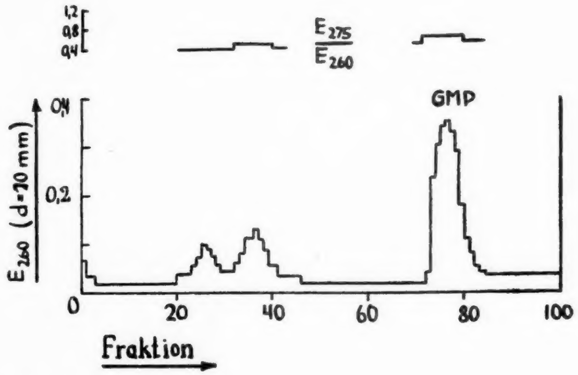


Abb. 8
Dowex—2, $HCOO^-$, 1.0×17.0 cm
Mischflasche: 450 ml H_2O
Reservoirflasche: 1—Ende 1 n $HCOONH_4$, 4 ml/Fraktion

dem sind durch die Rechromatographie zwei neue UV-Absorptionsmaxima sichtbar geworden: Die Fraktionen 88—96 enthalten eine noch nicht endgültig identifizierte Substanz, die auf Grund ihres Spektrums, des Verlaufs der Orzinreaktion [Abnahme der Extinktion bei 660 mμ nach verlängerter Hydrolyse, vgl. Hurlbert und Mitarbeiter (8)], möglicherweise als Purinnucleotid angesehen werden kann; der Phosphatgehalt beträgt etwa 1.4 M/M Ribose. Die zweite, noch unbekannte Verbindung, (Fraktionen 99—105) besitzt einen hohen E_{275}/E_{260}-Quotienten und wurde gleichfalls in anderen Tumoren und in der Leber normaler und tumortragender Tiere gefunden. Auf Grund des Verlaufes der Orzinreaktionen handelt es sich möglicherweise ebenfalls um ein Purinnucleotid.

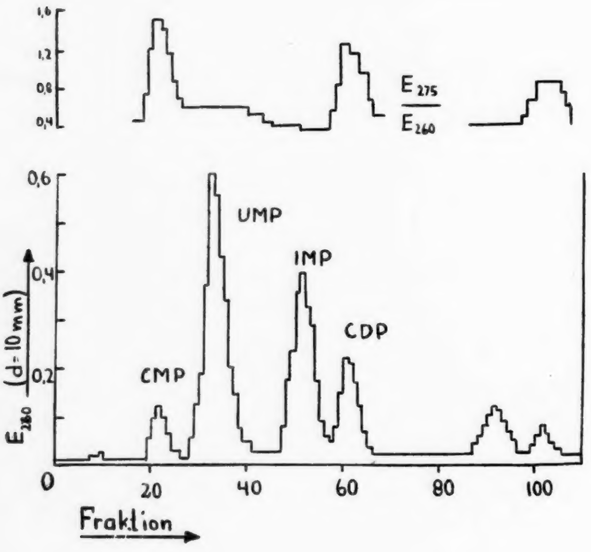


Abb. 9
Dowex—2, $HCOO^-$, 0.9 × 18.5 cm
Mischflasche: 475 ml H_2O
Reservoirflasche: 1—Ende 1 n $HCOONH_4$, 4 ml/Fraktion

Die Fraktionen 107—120 des S—1-Chromatogramms erwiesen sich bei der Rechromatographie (Abb. 10) als fast ausschliesslich aus Adenosin-5′-diphosphat bestehend. Das kleine UV-Absorptionsmaximum (Fraktionen 40—45) stellt eine geringe Menge — durch Aufarbeitung bis zur Rechromatographie — aus ADP entstandenes AMP dar. Die in den Fraktionen 118—124 und 137—145 angedeuteten UV-Absorptionsmaxima, welche durch einen hohen E_{275}/E_{260}-Quotienten gekennzeichnet sind, entsprechen den Fraktionen 114—125 und 134—142 der folgenden Abbildung.

Die sich an die ADP anschliessenden Fraktionen (121—132) der Abbildung 6 ergaben bei der Rechromatographie (Abb. 11) drei UV-Absorptionsmaxima. Das erste von ihnen (Fraktionen 102—109) entspricht dem im vorhergehenden S—2-Chromatogramm (Abb. 10) nicht erfassten Adenosin-5′-diphosphat, während die zwei folgenden (Fraktionen 114—125 und 134—142) noch nicht endgültig identifiziert sind. Anscheinend gleiche Verbindungen wurden in grösserer Menge aus säurelöslichen Extrakten von Hefen erhalten

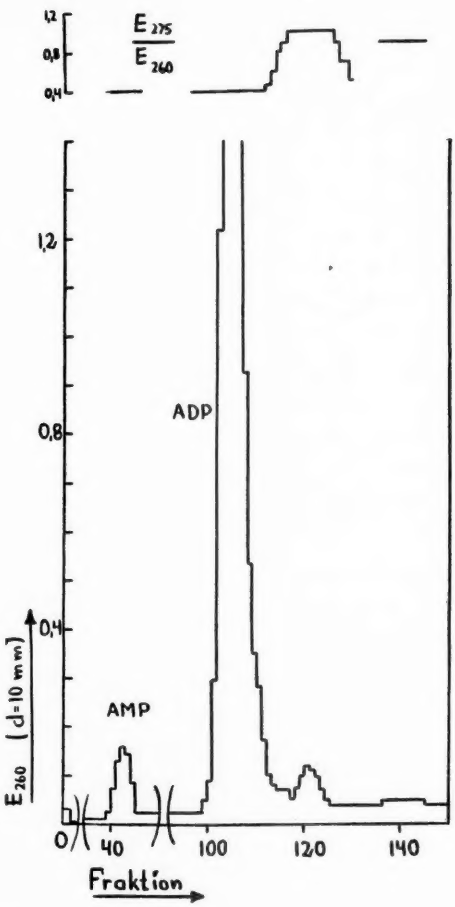


Abb. 10
Dowex—2, $HCOO^-$, 1.0×15.0 cm
Mischflasche: 380 ml H_2O
Reservoirflasche: 1—63 1 n $HCOONH_4$, 4 ml/Fraktion
64—Ende 2 n » , 5 ml/Fraktion

(vgl. Abb. 23 und 25) und die bisher an ihnen erhobenen Befunde sind dort zusammengefasst.

Die Rechromatographie (Abb. 12) der an S—1 als UDPA und UDPG bzw. UDPGa bezeichneten Fraktionen ergab keine weiteren Verbindungen. Die Fraktionen 27—33 enthalten Uridin-5′-diphosphat-N-acetylglucosamin (UDPA) und die Fraktionen 37—45 eine Mischung von Uridin-5′-diphosphat-glucose, bzw. -galactose. Beide UDP-Derivate wurden im Säugetiergewebe erstmalig von Hurlbert

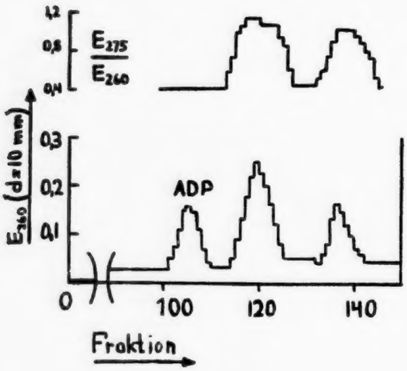


Abb. 11
Dowex—2, $HCOO^-$, 1.0×16.0 cm
Mischflasche: 375 ml H_2O
Reservoirflasche: 1—65 1 n $HCOONH_4$,
66—Ende 2 n » , 4 ml/Fraktion

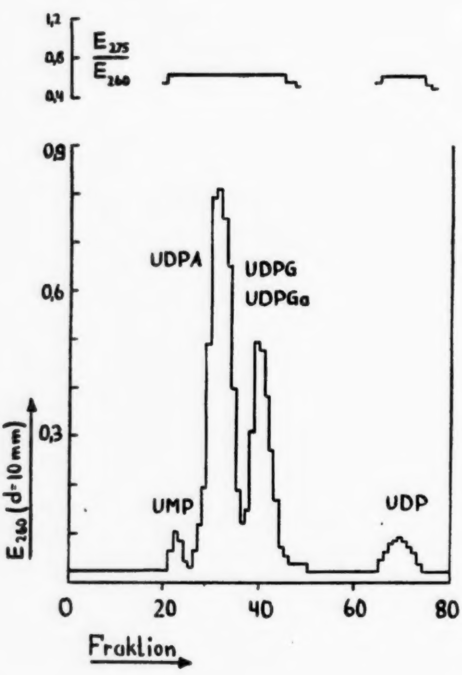


Abb. 12
Dowex—2, $HCOO^-$, 0.8×14.0 cm
Mischflasche: 430 ml H_2O
Reservoirflasche: 1—Ende 1 n $HCOONH_4$, 4 ml/Fraktion

(14, 15) sowie Hurlbert und Potter (17) gefunden, nachdem ihr Vorhandensein in bestimmten Hefen und Bakterien bereits früher von Leloir (7, 56), Leloir und Mitarbeitern (57) und Caputto und Mitarbeitern (58, 59) beschrieben worden war. In den Arbeiten von Smith und Mills (19, 60) konnte ebenfalls die Existenz dieser UDP-derivate in der Leber des Meerschweinchens sowie in dessen Milchdrüsengewebe nachgewiesen werden. Nach den bisherigen Untersuchungen dürfte es sich bei dem mit UDPG bzw. UDPGa bezeichneten UV-Absorptionsmaximum, um das bei der Umwandlung von Galactose in Glucose beteiligte Coferment handeln, das in der Literatur auch nach der durch es katalysierten Reaktion als Co-Galactowaldenase bezeichnet wird. Die beiden anderen UV-Absorptionsmaxima (Fraktionen 22—24 und 66—74) sind Abbauprodukte (UMP und UDP) der UDP-derivate, die durch Hydrolyse bei der Aufarbeitung entstanden sind.

Das sich an S—1 an die UDP-derivate anschliessende UV-Absorptionsmaximum wurde an S—2 rechromatographiert und die Ergebnisse sind in Abbildung 13 wiedergegeben. Die Hauptmenge erscheint in den Fraktionen 104—117 und konnte als Guanosin-5'-diphosphat identifiziert werden. Die Absorption der Fraktionen 42—47 ist durch Guanosin-5'-monophosphat, das durch Hydrolyse aus GDP gebildet wurde, bedingt. Bisher in Ratten- (8) und Mäuseleber, Niere (vgl. Abb. 27, 29 und 33) und einigen Hefen gefundene Adenosinderivate (Ad_x), welche etwa zwei M Phosphat/M Adenin enthalten, konnten ebensowenig in diesem wie in den anderen bisher untersuchten Tumoren gefunden werden.

Die Abbildung 14 repräsentiert das Rechromatogramm der Fraktionen 224—240 des S—1-Chromatogramms, deren erste durch einen erhöhten E_{275}/E_{260}-Quotienten gekennzeichnet sind. Die Rechromatographie zeigt zwei grössere UV-Absorptionsmaxima, die durch anschliessende Analysen als Uridin-5'-diphosphat und Cytidin-5'-triphosphat identifiziert wurden. Ein kleines, dem CTP folgendes UV-Absorptionsmaximum ist ebenfalls sichtbar. Die spektrophotometrischen und Riboseanalysen an diesen Fraktionen sprechen für ein Pyrimidinnucleotid. Möglicherweise handelt es sich um Uridin-5'-diphosphat-glucuronsäure, ein an der Glucuronidsynthese beteiligtes Coenzym [Dutton und Storey (61, 92)], das auch von Hurlbert (15), Hurlbert und Potter (17), Smith und Mills in der Leber (60) und von den letzteren Autoren ebenfalls im Milchdrüsen-

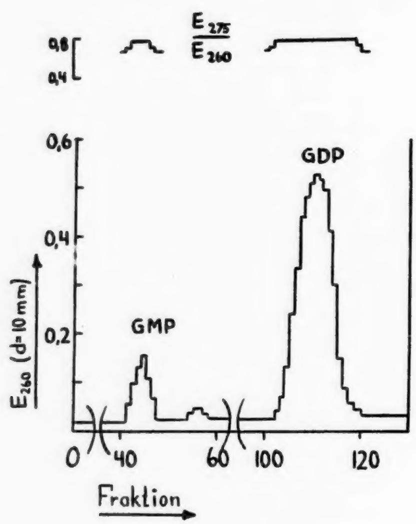


Abb. 13
Dowex—2, $HCOO^{-}$, 1.0×16.0 cm
Mischflasche: 450 ml H_2O
Reservoirflasche: 1—56 1 n $HCOONH_4$
57—Ende 2 n » , 3.9 ml/Fraktion

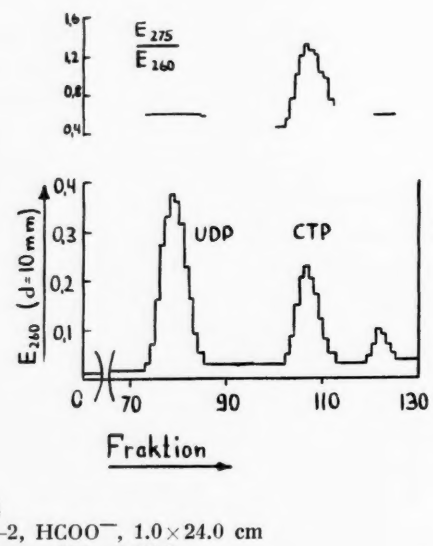


Abb. 14
Dowex—2, $HCOO^{-}$, 1.0×24.0 cm
Mischflasche: 510 ml H_2O
Reservoirflasche: 1—110 1 n $HCOONH_4$
111—Ende 2 n » , 4 ml/Fraktion

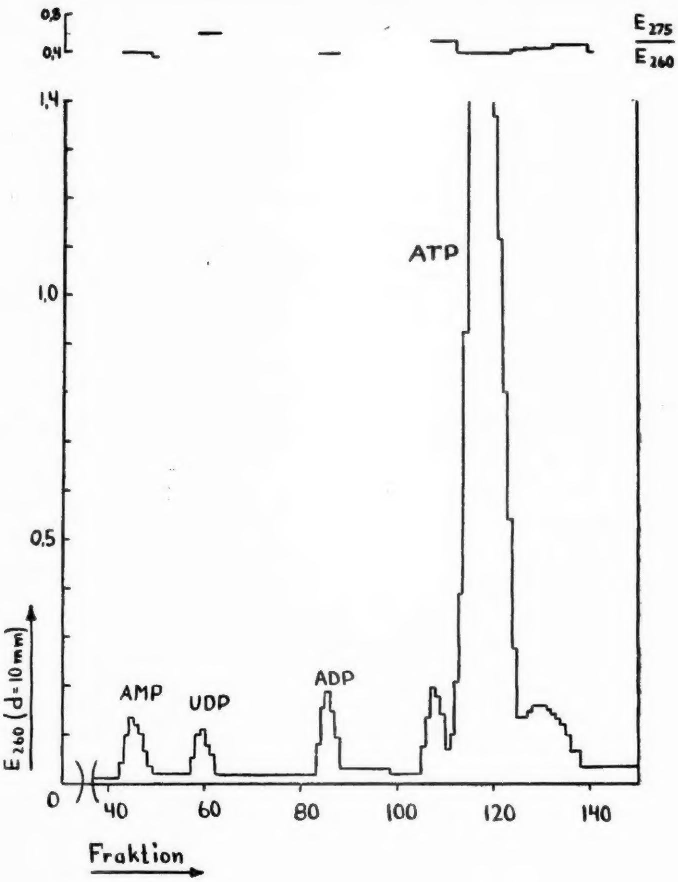


Abb. 15
Dowex—2, $HCOO^-$, 1.0 × 15.0 cm
Mischflasche: 350 ml H_2O
Reservoirflasche: 1—70 1 n $HCOONH_4$
71—Ende 2 n » , 4 ml/Fraktion

gewebe des Meerschweinchens (19) aufgefunden wurde. UDPGl kommt ferner in den Rattentumoren Flexner-Jobling-Carcinom (12) und Walker-256-Carcino-Sarkom (20) vor, während es in den bisher untersuchten Ascitestumoren Yoshida (vgl. Abb. 20), Ehrlich (21) und Sarkom — 37 (23) nicht sicher nachgewiesen werden konnte. Obwohl in den hier beschriebenen Untersuchungen für die Herstellung der säurelöslichen Extrakte von etwa gleichen Gewebemengen wie bei den Flexner-Jobling-, Walker- und Jensen-Tumoren

ausgegangen wurde, konnten keine, für eine sichere Identifizierung ausreichenden Mengen dieses UDP-derivates erhalten werden.

In der Reihe der rechromatographierten Sektionen der Abbildung 6 folgen die Fraktionen 241—253. In Abbildung 15 erkennt man ein grosses (Fraktionen 112—125) und fünf kleine (Fraktionen 43—48, 58—62, 84—88, 106—110 und 126—138) UV-Absorptionsmaxima: Adenosin-5'-triphosphorsäure und durch ihre Aufarbeitung entstandene ADP und AMP, im vorhergehenden Rechromatogramm nicht erfasste UDP und zwei noch unbekannte, ATP einschliessende Verbindungen.

Die Rechromatographie wird beendet mit den GTP—UTP-Fraktionen der Abbildung 6. Als Hauptbestandteile treten wiederum Guanosin- und Uridin-5'-triphosphat sowie eine kleine Menge ihrer

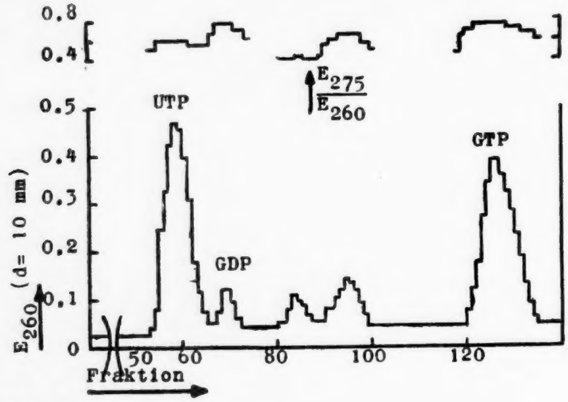


Abb. 16
Dowex—2, $HCOO^-$, 1.0×20.0 cm
Mischflasche: 500 ml H_2O
Reservoirflasche: 1—65 1 n $HCOONH_4$
66—95 2 n »
96—Ende 2 n » +0.25 n HCOOH,
3.9 ml/Fraktion

durch die Aufarbeitung entstandenen Spaltprodukte auf (Abbildung 16). Ausserdem finden wir zwei kleine UV-Absorptionsmaxima (Fraktionen 81—87 und 91—99), die noch nicht endgültig identifizierte Verbindungen enthalten. Bei dem ersteren von ihnen handelt es sich möglicherweise um ein phosphoryliertes Adenosinderivat, wofür die Ergebnisse der spektrophotometrischen Auswertung und der Verlauf der Orzinreaktion sprechen. Die durch einen erhöhten E_{275}/E_{260}-Quotienten gekennzeichneten Fraktionen

91—99 lieferten nach Entfernung des Eluierungsmittels nicht genügend Material, um eingehende spektrophotometrische und sonstige Analysen zu ermöglichen.

β) Mononucleotide aus den Nucleinsäuren des Yoshida-Sarkoms

Die Chromatographie (Abb. 17) der durch alkalische Hydrolyse aus den Ribonucleinsäuren des Yoshida-Sarkoms entstandenen Mononucleotide an S—1 ergab eine gute Auftrennung der Cytidin-, Adenosin- und Guanosin — 2'- und 3'-phosphorsäureester, während die Trennung der Isomeren der Uridylsäure nur unvollständig gelang. Ähnliche Schwierigkeiten bei der Auftrennung der 2'- und 3'-Phosphorsäureester des Uridins wurden bereits von Cohn (50) festgestellt.

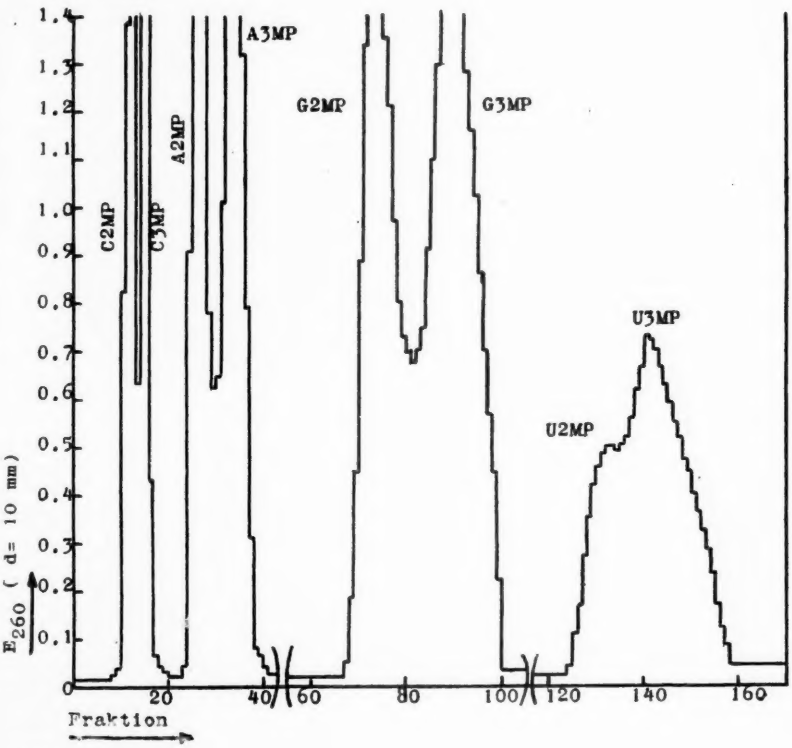


Abb. 17
Dowex—1, $HCOO^-$, 1.0 × 22.0 cm
Mischflasche: 500 ml H_2O
Reservoirflasche: 1—50 1 n HCOOH
51—Ende 3 n » , 2.5 ml/Fraktion

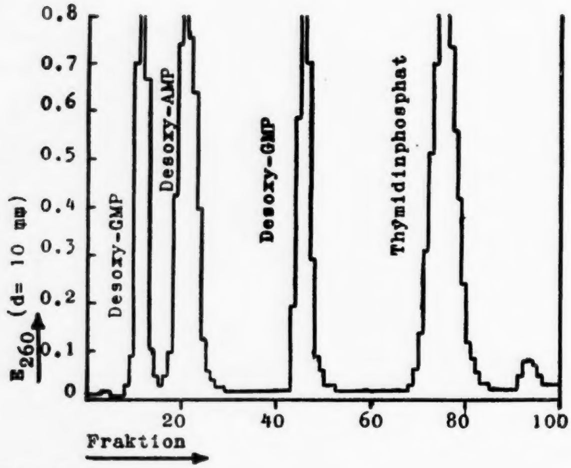


Abb. 18
Dowex—2, $HCOO^-$, 1.0×22.0 cm
Mischflasche: 600 ml H_2O
Reservoirflasche: 1—Ende 3 n HCOOH, 4 ml/Fraktion

Die Chromatographie der durch enzymatischen Abbau aus Desoxyribonucleinsäure gebildeten Desoxymononucleotide (Abb. 18) wurde unter den für Ribonucleotide beschriebenen Bedingungen durchgeführt. Die Desoxymononucleotide von Adenin, Guanin und Cytosin nehmen die gleichen Stellen am Chromatogramm ein, wie die entsprechenden 5′-Ribonucleotide, während sich Thymidinphosphat wie Uridin-5′-monophosphat verhält.

b) Jensen-Sarkom

α) Säurelöslicher Extrakt

Die Auftrennung des säurelöslichen Extraktes aus dem Jensen-Sarkom (Abb. 19) an S—1 ergab eine weitgehende Übereinstimmung in seiner Zusammensetzung mit dem festen Yoshida-Sarkom (vgl. Abb. 6). Auch aus dem Jensen-Sarkom konnten durch Chromatographie an S—1 und S—2 und durch anschliessende Analysen die 5′-Mono-, Di- und Triphosphorsäureester von Adenosin, Guanosin, Cytidin und Uridin sowie die Uridin-5′-diphosphatderivate UDP-N-acetylglucosamin, UDP-glucose bzw. -galactose und UDP-glucuronsäure gewonnen werden.

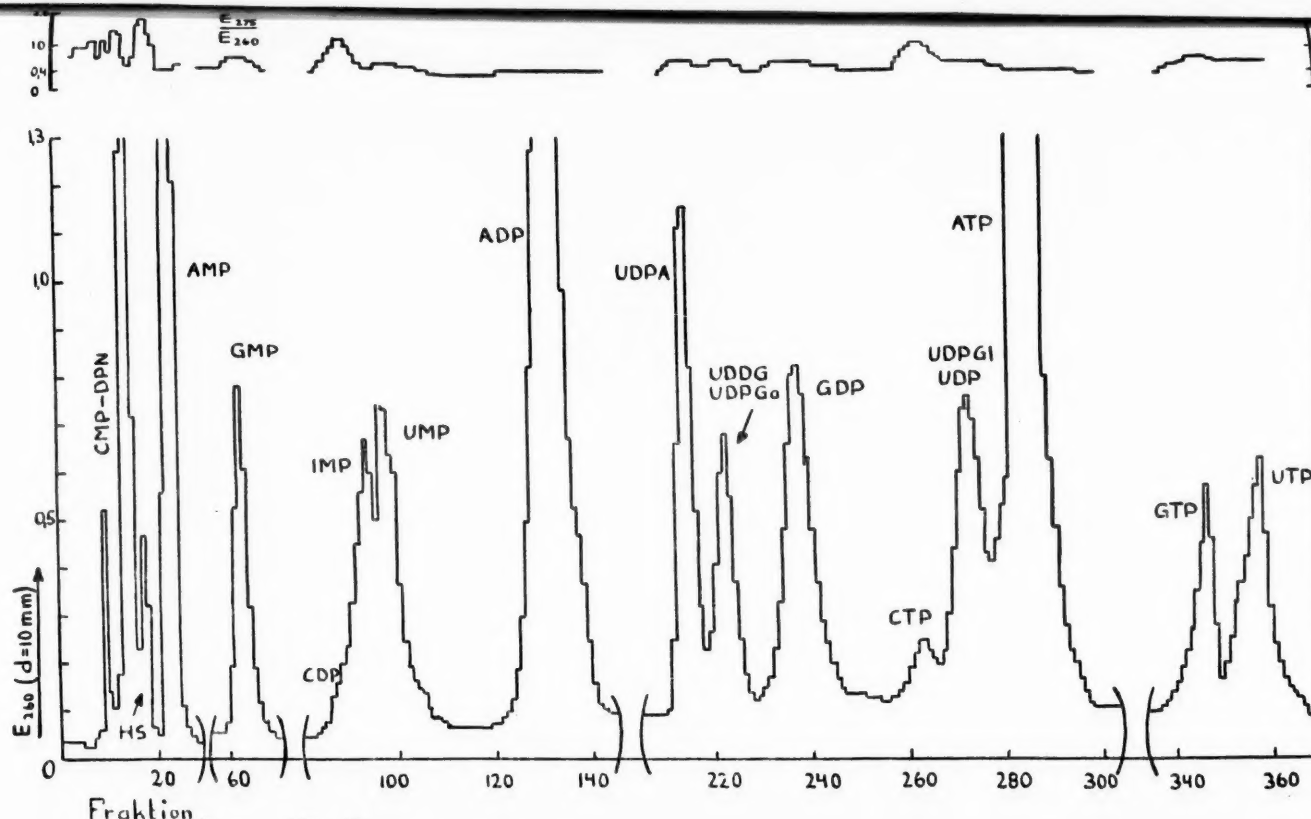


Abb. 19
Dowex—2, $HCOO^-$, 1.1×22.0 cm
Mischflasche: 500 ml H_2O
Reservoirflasche: 1—145 3 n HCOOH
146—260 4 n » +0.4 n $HCOONH_4$
261—Ende 4 n » +0.8 n »
1—260 4.2 ml/Fraktion, 261—Ende 3 ml/Fraktion

β) Nucleinsäuren des Jensen-Sarkoms

In qualitativer und quantitativer Hinsicht entsprechen die Bestandteile der Ribo- und Desoxyribonucleinsäuren des Jensen-Sarkoms unter den hier beschriebenen Untersuchungsbedingungen denen des Yoshida-Tumors.

c) Walker-256-Carcino-Sarkom und Flexner-Jobling-Carcinom

Die Ergebnisse der mittels Ionenaustauschchromatographie durchgeführten Trennungen der freien und polymerisierten Nucleotide dieser Rattentumoren entsprachen weitgehend den am Yoshida-Tumor und am Jensen-Sarkom erhobenen Befunden. Die Abbildungen der Chromatogramme wurden fortgelassen, da bereits repräsentative Darstellungen beschrieben worden sind (20, 12, 13). Die nochmalige Untersuchung dieser Tumoren wurde vorgenommen, um die Unterlagen für einen Vergleich zwischen wachsenden und ruhenden Geweben zu vermehren.

2.) ASCITESTUMOREN

a) Zellen des Yoshida-Sarkoms

Als Vertreter aus der heute für biochemische Untersuchungen grosses Interesse beanspruchenden Gruppe der Ascitestumoren ist in Abbildung 20 der Gehalt der Ascitesform des Yoshida-Tumors an freien Nucleotiden wiedergegeben. Zur Untersuchung dienten die Zellen (6.5 g, Feuchtgewicht) der Tumoren von fünf Tieren am siebenten Tage nach der Übertragung.

Obwohl das S—1-Chromatogramm dieses Tumors, wie das der anderen untersuchten Gewebe, in Sektionen rechromatographiert worden ist, wurde auf eine graphische Wiedergabe der S—2-Chromatogramme verzichtet, da die Ergebnisse im Prinzip denen in Abbildungen 7—16 wiedergegebenen entsprechen.

b) Zellen von Sarkom-37, Ehrlich (diploid), Ehrlich-Colchicinresistent (62) und Ehrlich-»Wärmestamm» (63)

Weder der freie Nucleotidgehalt noch die Zusammensetzung der Nucleinsäuren dieser Ascitestumoren unterscheiden sich wesentlich von den an der Ascitesform des Yoshida-Sarkoms gefundenen

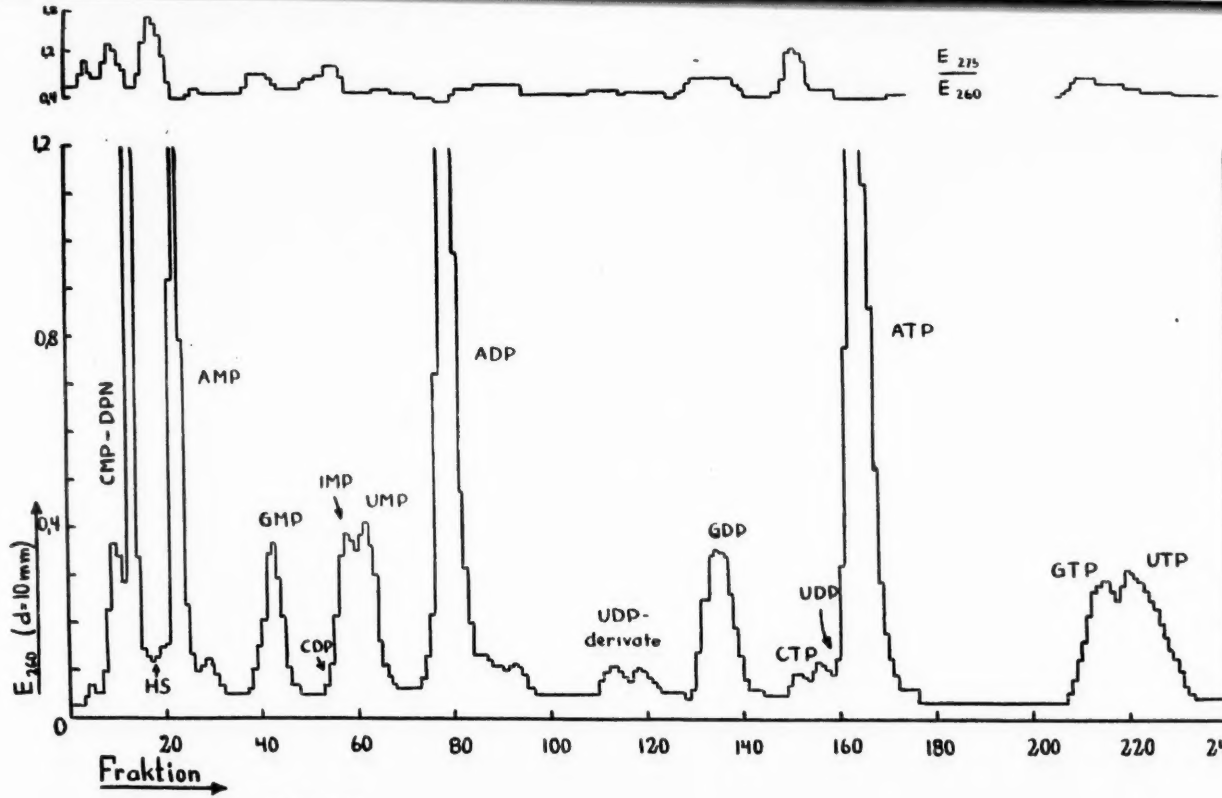


Abb. 20
Dowex—2, $HCOO^-$, 0,8 × 14,0 cm
Mischflasche: 320 ml H_2O

Reservoirflasche:	1—30	1 n HCOOH			
	31—95	4 n »			
	96—175	4 n »	+0,4 n $HCOONH_4$		
	176—Ende	4 n »	+0,8 n	»	4 ml/Fraktion

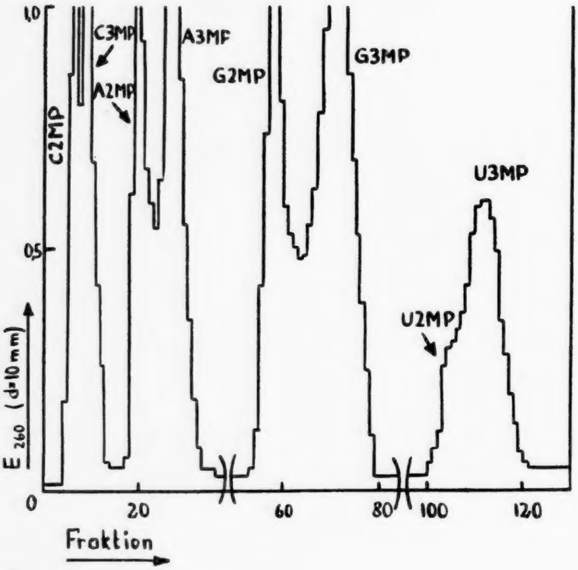


Abb. 21a
Dowex—2, $HCOO^-$, 0.9×14 cm
Mischflasche: 315 ml H_2O
Reservoirflasche: 1—37 1 n HCOOH
38—Ende 3 n » , 3.6 ml/Fraktion

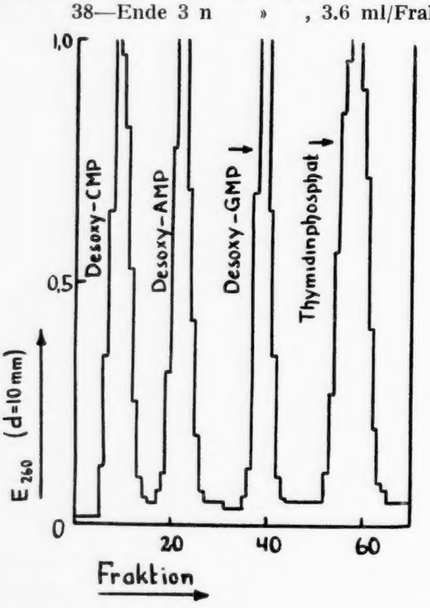


Abb. 21b
Dowex—2, $HCOO^-$, 1.1×20.0 cm
Mischflasche: 500 ml H_2O
Reservoirflasche: 1—Ende 3 n HCOOH, 4.2 ml/Fraktion

Werten, und es wurde daher von einer graphischen Darstellung dieser Ergebnisse abgesehen, zumal S—1-Chromatogramme der säurelöslichen Extrakte aus Ehrlich-diploid und Sarkom-37 (21, 23) vorliegen.

c) Mononucleotide aus den Nucleinsäuren der Ascitestumoren

Die chromatographischen Auftrennungen der durch alkalische von RNS und durch enzymatische Hydrolyse von DNS aus den Zellen der Ascitesform des Yoshida-Sarkoms gewonnenen Mononucleotide (Abb. 21 a und b) zeigten keine bemerkenswerten Unterschiede in der Zusammensetzung der Nucleinsäuren zwischen der festen und der Ascitesform. Analoge Befunde wurden über die Zusammensetzung der Nucleinsäuren der Ascitestumoren Sarkom-37, Ehrlich (diploid), Ehrlich-Colchicin-resistent und Ehrlich-»Wärmestamm» erhalten.

3.) SERA DER ASCITESTUMOREN

Die Abbildung 22 zeigt die säulenchromatographische Auf-

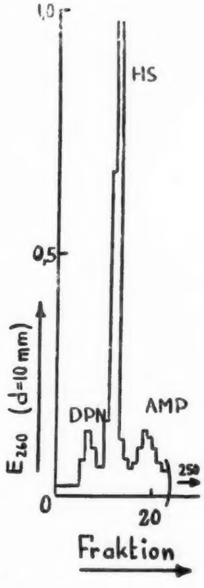


Abb. 22

Dowex—2, $HCOO^-$, 0.9 × 11.0 cm
Mischflasche: 250 ml H_2O
Reservoirflasche: 1—Ende 4 n HCOOH, 4 ml/Fraktion

trennung der zellfreien Ascitesflüssigkeit des Yoshidatumors. Als Bestandteile konnten in dem säurelöslichen Extrakt lediglich Harnsäure, kleine Mengen an DPN und Spuren von AMP gefunden werden.

Die Sera der Ascitestumoren Sarkom-37, Ehrlich (diploid), Ehrlich-Colchicin-resistent und Ehrlich-»Wärmestamm» wiesen die gleiche qualitative Zusammensetzung ihrer im ultravioletten Bereich absorbierenden Verbindungen auf.

Polymerisierte Nucleinsäuren konnten aus den Sera der Ascitestumoren nicht isoliert werden.

4.) HEFEZELLEN

a) Chromatographie der säurelöslichen Extrakte

Als ein Beispiel für die Isolierung von freien Nucleotiden aus Hefezellen stellt die Abbildung 23 die Auftrennung des gesamten säurelöslichen Extraktes aus 25 g Hefe dar (Firma Dr. Fritz Hillringhaus, Wuppertal-Barmen). Das S—1-Chromatogramm lässt erkennen, dass auch in Hefezellen neben den 5′-Mono-, Di- und Triphosphorsäureestern des Adenosins die analogen Ester von Guanosin, Cytidin, Uridin und UDP-derivate enthalten sind [vgl. (22)]. Aus Leloir's Arbeitskreis, dem wir die Entdeckung der freien Urazilnucleotide und verschiedener ihrer Derivate verdanken, wurde bereits früher das Vorkommen von einigen freien 5′-Nucleotiden (AMP, ADP, IMP und GMP) beschrieben [Cabib und Mitarbeiter (64)].

Die Isolierung und Identifizierung der in Abbildung 23 genannten Verbindungen erfolgten unter den für das Yoshida-Sarkom beschriebenen Bedingungen: zweifache Chromatographie, Bestimmung von Phosphat, Ribose, Spectrum.

Aus der Reihe der an diesem Hefechromatogramm durchgeführten Rechromatographieen soll zunächst die Isolierung der an sich bekannten, aber nur in sehr wenigen Geweben nachgewiesenen Guanosin-5′-diphosphorsäure-mannose erörtert werden, die bisher in keinem der hier untersuchten Gewebe gefunden werden konnte.

Das am S—1-Chromatogramm der Hefe (Abb. 23) als GDPM bezeichnete UV-Absorptionsmaximum wurde an S—2-rechromatographiert (Abb. 24). Die Fraktionen 59 bis 70 zeigten eine Verbindung, deren Spektrum auf ein Guanin-Nucleotid hinwies. Da in

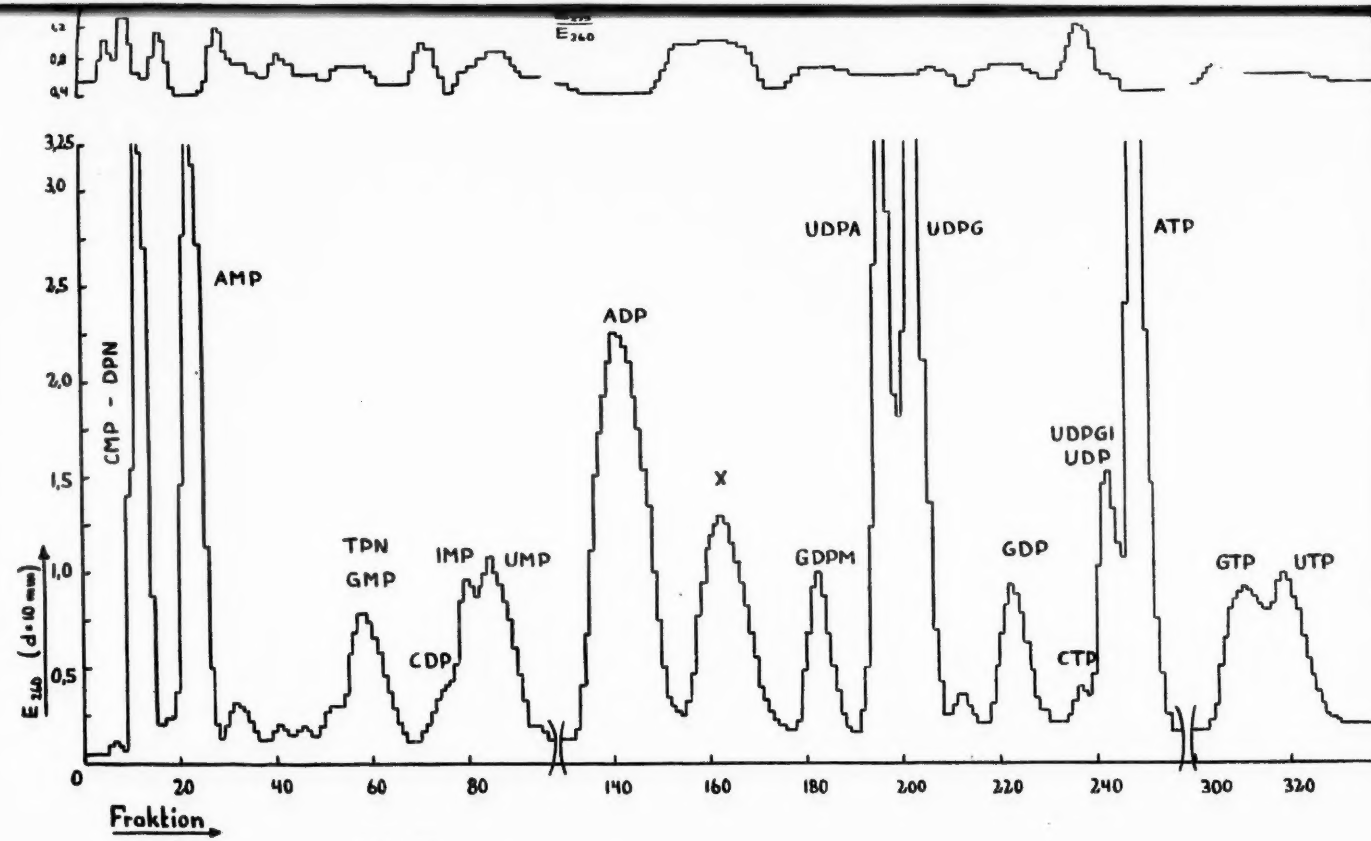


Abb. 23
Dowex—2, $HCOO^-$, 1.0×20.0 cm
Mischflasche: 485 ml H_2O

Reservoirflasche:	1—163	3 n HCOOH	
	164—257	3 n »	$+0.4$ n $HCOONH_4$
	258—Ende	4 n »	$+0.8$ n » , 4.1 ml/Frakt.

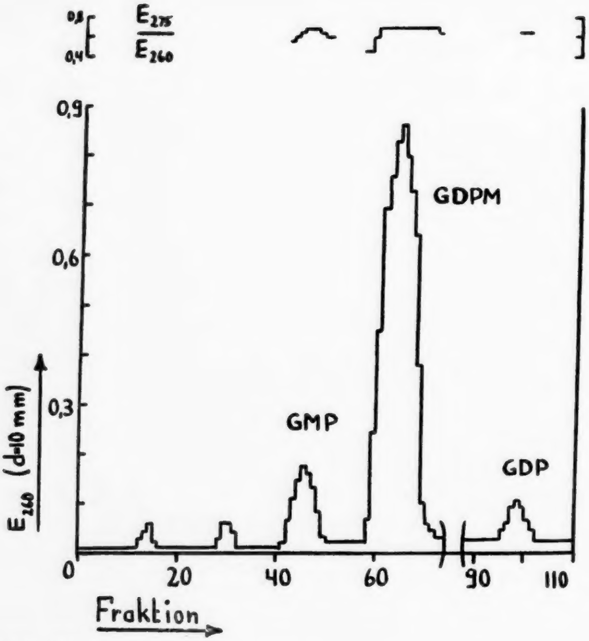


Abb. 24
Dowex—2, $HCOO^-$, 1.0×16.0 cm
Mischflasche: 450 ml H_2O
Reservoirflasche: 1—50 1 n $HCOONH_4$
51—Ende 2 n » , 4 ml/Fraktion

dem gleichen Chromatogramm geringe Mengen an GMP und GDP gefunden wurden, lag die Vermutung nahe, dass es sich um ein Derivat von Guanosin-5′-diphosphat handeln könnte. Die Analysen ergaben, dass die Substanz zwei M Phosphat pro M Guanin enthält. Durch saure Hydrolyse in 0.5n HCl bei 60° nach Kita und Peterson (65) wurde Mannose abgespalten und nach Gurin und Hood (66) identifiziert. Diese Ergebnisse rechtfertigen die Annahme, dass es sich bei der als GDPM bezeichneten Verbindung um die von Cabib und Leloir (67) entdeckte Guanosin-5′-diphosphorsäure-mannose handelt. Strominger (68) berichtete ebenfalls über zwei ähnliche Verbindungen in dem säurelöslichen Extrakt des Hühnereileiters, die er als Guanosin-5′-pyrophosphat-mannose bezeichnete.

Diese, wie auch die anderen untersuchten Hefen, enthielten ausser den oben erwähnten Nucleotiden zahlreiche, im ultravioletten Bereich absorbierende Verbindungen in unterschiedlichen, meistens

recht geringen, Konzentrationen und vorläufige Befunde zeigen an, dass es sich bei einigen von ihnen um noch unbekannte Nucleotide handelt.

b) Untersuchungen über neue, im ultravioletten Bereich absorbierende Verbindungen

Bei einem Vergleich der Abbildung 23 mit den S—1-Chromatogrammen aus Säugetiergeweben und Tumoren fällt neben dem durch GDPM bedingten das sich der ADP anschliessende UV-Absorptionsmaximum (Fraktionen 156—174) durch seine Grösse besonders auf, da es in den anderen Geweben entweder nicht oder nur angedeutet erkennbar ist.

Die Rechromatographie der Fraktionen 146—174 machte, neben den durch die Adenosin-phosphorsäureester (AMP und ADP) bedingten, noch fünf weitere UV-Absorptionsmaxima sichtbar, welche durch keine bisher bekannten Nucleotide erklärt werden konnten. Um für eine mögliche Identifizierung dieser Verbindungen eine grössere Menge an Ausgangsmaterial zu haben, wurden wiederholt grössere Portionen dieser Hefe extrahiert und an S—1 und S—2 chromatographiert; die Abbildung 25 zeigt ein S—2 Chromatogramm aus vergleichbaren Fraktionen eines solchen S—1-Chromatogramms.

In dieser Abbildung sind die oben erwähnten, durch noch unbekannte Verbindungen bedingten UV-Absorptionsmaxima als X_1 (Fraktionen 11—17), X_2 (Fraktionen 21—26), X_3 (Fraktionen 54—63), X_4 (Fraktionen 87—105) und X_5 (Fraktionen 110—135) bezeichnet. Ausserdem lassen die Änderungen der E_{275}/E_{260}-Quotienten der Fraktionen 27—35 die Anwesenheit einer weiteren Verbindung vermuten.

Aus der Reihe dieser unbekannten Verbindungen wurden X_1, X_2, X_4, X_3 und X_5 auf ihre Fähigkeit, ultraviolettes Licht zu absorbieren und ihre Gehalte an Phosphat und Ribose untersucht[1]. Von den durch die Gefriertrocknung erhaltenen Rückständen wurden Proben in 0.001 nHCl bzw. 0.1 n NaOH aufgenommen und ihre Absorption im ultravioletten Bereich mit Hilfe eines automatisch-registrierenden Spektralphotometers (Beckman, Modell DK—1) gemessen. Die

[1] Die mit X_5 bezeichnete Verbindung wurde bereits vor längerer Zeit von Dr. H. Schmitz, Marburg/L., in einigen säurelöslichen Extrakten gefunden. Eine gemeinsame Veröffentlichung der Ergebnisse ist in Vorbereitung (71).

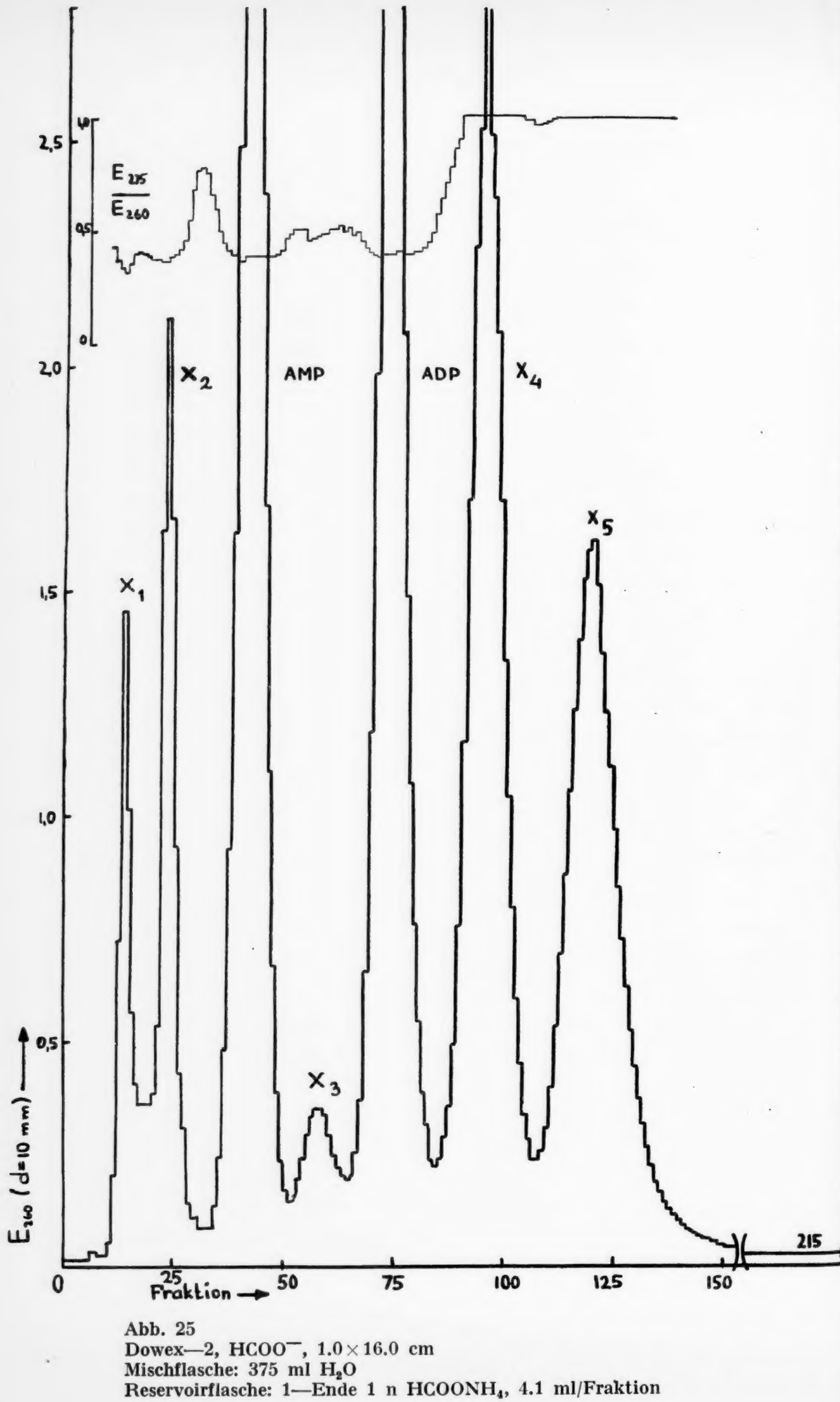


Abb. 25
Dowex—2, $HCOO^-$, 1.0 × 16.0 cm
Mischflasche: 375 ml H_2O
Reservoirflasche: 1—Ende 1 n $HCOONH_4$, 4.1 ml/Fraktion

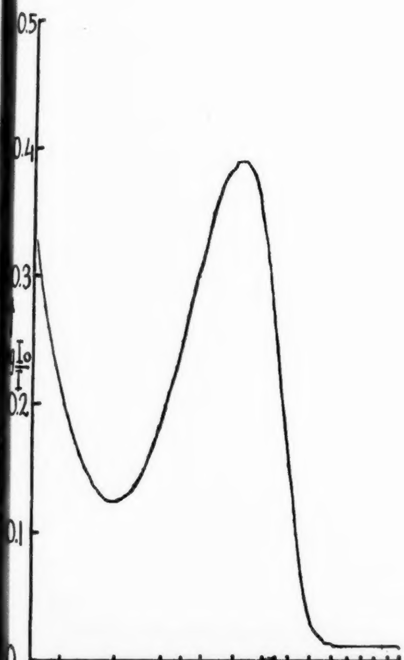


Abb. 26a
Beckman-DK-1 Spektrogramm von X_1
Lösungsmittel: 0.001 n HCl
Lichtweg: 1 cm

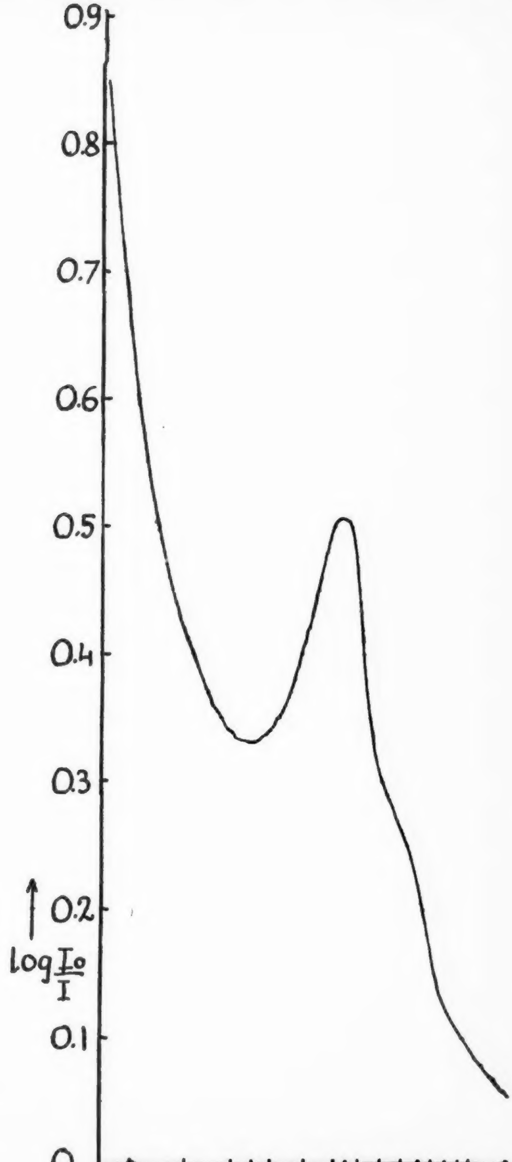


Abb. 26b
Beckman-DK-1 Spektrogramm von X_2 [1]

Lösungsmittel: 0.001 n HCl
Lichtweg: 1 cm

[1] Der nach Gefriertrocknung in 0.001n HCl aufgenommene Rückstand war stark gelb gefärbt.

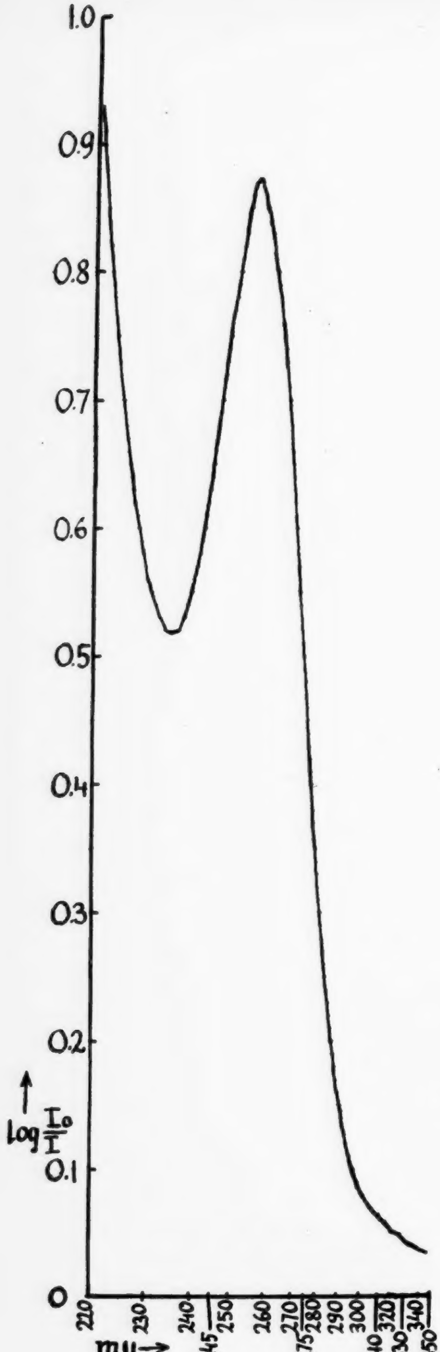


Abb. 26c
Beckman-DK-1 Spektrogramm von X_3
Lösungsmittel: 0.001 n HCl
Lichtweg: 1 cm

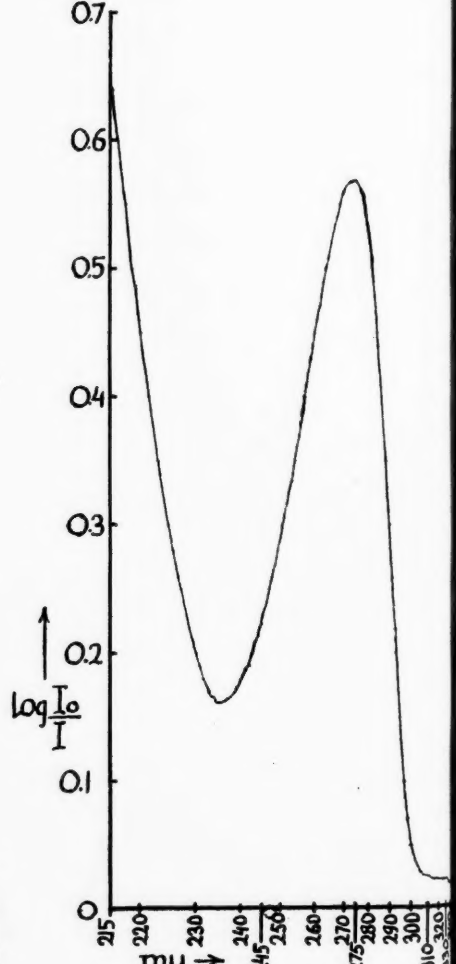


Abb. 26d
Beckman-DK-1 Spektrogramm von X
Lösungsmittel: 0.001 n HCl
Lichtweg: 1 cm

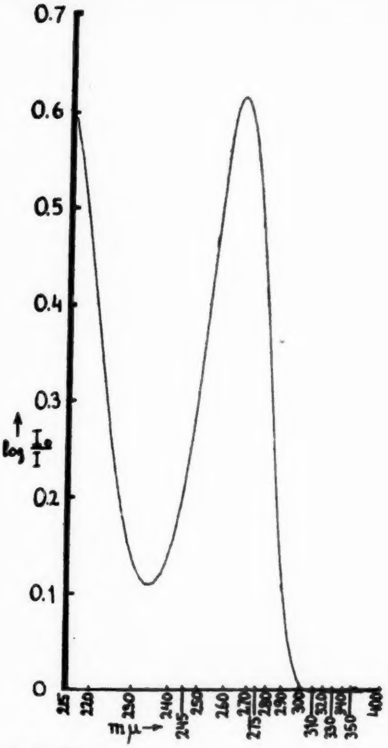


Abb. 26e
Beckman-DK-1 Spektrogramm von X_5
Lösungsmittel: 0.001 n HCl
Lichtweg: 1 cm

Ergebnisse der spektrophotometrischen Auswertungen im sauren Milieu sind in Abbildung 26 a—e wiedergegeben; weitere, im sauren und alkalischen Milieu ermittelte Werte sind in Tabelle 1 aufgeführt.

Die vorgelegten spektrophotometrischen Ergebnisse stimmen nicht mit den an — bisher als natürlich vorkommend — bekannten Nucleotiden oder an deren Abbauprodukten (Nucleoside, Purin- und Pyrimidinbasen) erhaltenen überein.

Die Analysen auf die Gehalte an Ribose und Phosphat von X_1, X_2, X_3, X_4 und X_5 erbrachten die in Tabelle 1 verzeichneten Werte. Als Bezugsgrösse wurde 1 E_{260}-Einheit gewählt, die der Extinktion von 1.00/ml Lösung in 0.001nHCl bei 260 mμ (d = 10 mm) entspricht. Weder die Ergebnisse der Ribose — noch die der Phosphatanalysen zeigten eine Übereinstimmung mit an bekannten

TABELLE 1

Verbindung	pH	Wellenlänge		Optische Dichte bei einer gegeb. Wellenlänge / Optische Dichte des Maximums in Säure							Offensichtl. Ribose* / Hydrolysedauer		Gesamtphosphat*
		Maximum	Minimum	Maximum	Minimum	230 mμ	245 mμ	260 mμ	275 mμ	290 mμ	15 min.	60 min.	
X_1	H^+	261	230	1.000	0.295	0.270	0.660	1.000	0.655	0.077	0.014	0.013	0.010
	OH^-	268	240	0.925	0.380	0.819	0.420	0.795	0.777	0.056			
X_2	H^+	272	244	1.000	0.653	0.785	0.653	0.830	0.985	0.580	0.037	0.041	0.108
	OH^-	275	245	0.985	0.564	0.914	0.564	0.670	0.985	0.549			
X_3	H^+	257	235	1.000	0.596	0.630	0.755	0.960	0.590	0.175	0.170	0.162	0.174
	OH^-	260	222	0.870	0.139	0.217	0.525	0.870	0.445	0.076			
X_4	H^+	273	236	1.000	0.279	0.332	0.415	0.820	0.980	0.440	0.023	0.026	0.022
	OH^-	274	240	1.038	0.351	0.611	0.386	0.735	1.019	0.211			
X_5	H^+	267	234	1.000	0.158	0.188	0.336	0.910	0.986	0.153	0.058	0.058	0.056
	OH^-	268	234	1.070	0.136	0.151	0.311	0.905	0.881	0.191			
AMP		**									0.068	0.060	0.068
GMP		**									0.072	0.063	0.072
CMP		**									0.000	0.001	0.037
UMP		**									0.016	0.046	0.101

* Angaben in $\mu M/E_{260}$-Einheit.
** vgl. Tabelle 2, S. 64.

Substanzen ermittelten Werten, wie — unter Berücksichtigung der in Tabelle 2 angegebenen Extinktionskoeffizienten — aus den Tabellen 3 und 4 errechnet werden kann.

Das Vorliegen von Nucleosid-pyrophosphorsäureestern ist nicht anzunehmen, da nach 15—30 min Hydrolyse in 1 n HCl (100°) keine — in Bezug auf das Gesamtphosphat kennzeichnenden Mengen — an anorganischem Phosphat nachweisbar waren. Dieser Befund lässt auch die Möglichkeit des Vorliegens von zweifach phosphorylierten Nucleosiden (2′-, 5′- oder 3′-, 5′-) als unwahrscheinlich erscheinen [vgl. Cohn und Volkin (70)].

Weder die erwähnten noch die folgenden Untersuchungen, nämlich wiederholte Chromatographie an basischen Austauschern, Adsorption an Tierkohle mit nachfolgender Eluierung durch 10- und 25%iges Pyridin, Hydrolyse der unbekannten Verbindungen durch 11.5 n Perchlorsäure (43, 44) und anschliessender Chromatographie der Hydrolysate an Kationenaustauschsäulen [Dowex-50, H^+ nach Cohn (49)] ermöglichten eine klare Definierung dieser neuen Verbindungen.

Obwohl die in Frage stehenden Basen noch nicht endgültig identifiziert werden konnten, dürften die in Tabelle 1 zusammengefassten Ergebnisse dafür sprechen, dass es sich bei den Verbindungen X_1, X_2, X_3, X_4 und X_5 um Nucleotide handelt: Absorption in den für Nucleotide typischen Bereichen des ultravioletten Lichtes, Gehalte an Ribose und an Phosphorsäure. Das Vorliegen von aliphatischen Ribotiden [Glycinamid-ribotid oder (*a-N*-Formyl)-glycinamid-ribotid, (80)] dürfte auszuschliessen sein, da diese unter den hier beschriebenen Bedingungen — entsprechend ihrer Ladung — bereits früher von dem basischen Ionenaustauscher eluiert worden wären; ausserdem unterscheiden sich ihre UV-Spektra von den hier für X_1—X_5 angegebenen (93). Die mögliche Übereinstimmung von X_5 mit der von Carter und Cohen entdeckten Adenylbernsteinsäure konnte bisher nicht sichergestellt werden.

5.) RUHENDE GEWEBE

a) Leber

In der Abbildung 27 ist ein S—1-Chromatogramm aus dem Extrakt des Lebergewebes von drei normalen Mäusen dargestellt. Obwohl auch Lebergewebe von tumortragenden Tieren untersucht wurde, konnten zwischen diesem und demjenigen aus tumorfreien Tieren keine Unterschiede festgestellt werden. Die hier nicht dargestellten Chromatogramme aus den Lebern von tumortragenden Mäusen boten das gleiche Bild wie die aus Lebern von normalen und Tumorratten (8, 12).

Ein Vergleich des Leberchromatogramms mit den korrespondierenden Chromatogrammen des Yoshida-Tumors und auch der anderen festen Tumoren (vgl. Abb. 6 und 19) zeigt einige Unterschiede in qualitativer und quantitativer Hinsicht: Die den GMP-Fraktionen des Tumors entsprechenden Fraktionen der Leber weisen zusätzlich die Beschriftung TPN auf. Aus diesen Fraktionen der Leber konnten bei der Rechromatographie (Abb. 28) an S—2 genügende Mengen des Cofermentes isoliert werden, die eine Charakterisierung durch folgende Analysen erlaubten: Spektrum, Ribose- und Phosphatbestimmung, KCN-Reduktionstest (69).

Ein quantitativer Unterschied zwischen den Extrakten der Leber und denen der Tumoren besteht darin, dass erstere mehr Inosin-5'-phosphat aufweisen.

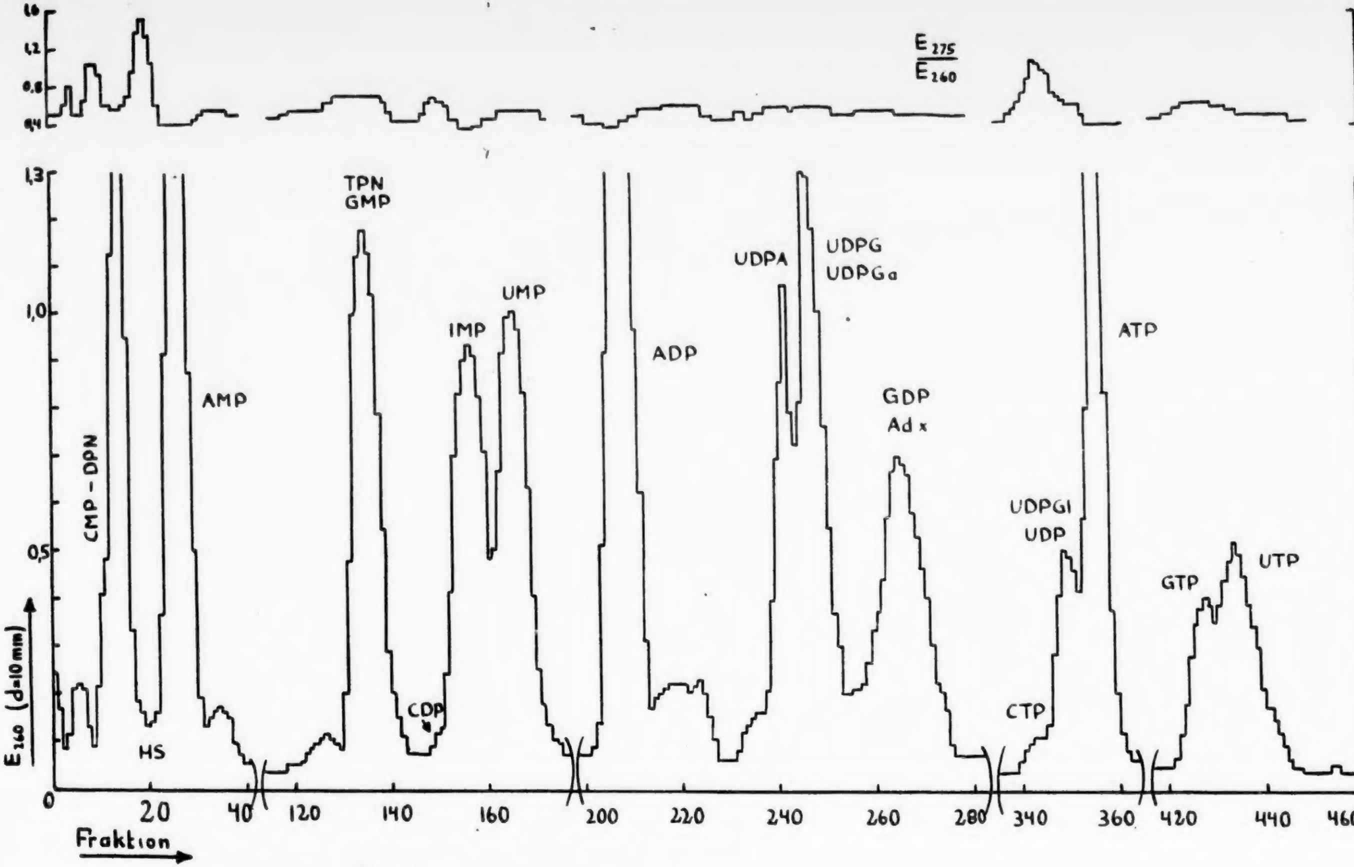


Abb. 27
Dowex—1, $HCOO^-$, 1.0×18 cm
Mischflasche: 480 ml H_2O
Reservoirflasche: 1—109 1 n HCOOH, 5 ml/Fraktion
110—201 4 n »
202—270 4 n » + 0.2 n $HCOONH_4$, 4 ml/Fraktion

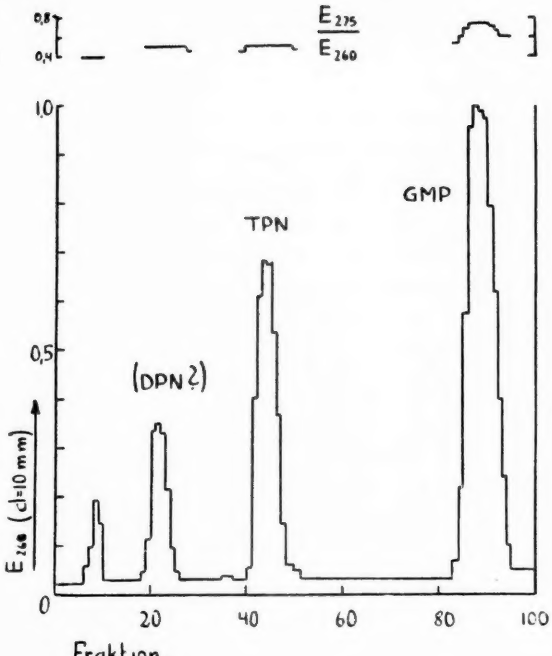


Abb. 28
Dowex—2, $HCOO^-$, 1.0×16 cm
Mischflasche: 475 ml H_2O
Reservoirflasche: 1—Ende 1 n $HCOONH_4$, 4 ml/Fraktion

Im Gegensatz zu den Tumorchromatogrammen folgen in denen aus Mäuseleber — wie bei Ratten (8, 12) — den UDP-derivaten UDPA und UDPG bzw. UDPGa Fraktionen, die nicht nur Guanosin-5′-diphosphat, sondern auch Ad_x enthalten. Während die Isolierung und die Identifizierung der GDP keine Schwierigkeiten bereitete, gelang es bisher nicht, eine völlige Identifizierung der Ad_x-Verbindungen durchzuführen. Die Rechromatographie dieser Fraktionen (Abb. 29) zeigt, dass sich dieses UV-Absorptionsmaximum aus mindestens fünf Komponenten zusammensetzt. Bei der Bestimmung des labilen Phosphates wurden keine signifikanten Mengen an anorganischem Phosphat freigesetzt. Erst nach längerer Einwirkung von 1 nHCl (100°) wurden grössere Mengen an anorganischem Phosphat frei, die in ihrer Gesamtmenge etwa den Ergebnissen entsprechen, die Cohn und Volkin (70) unter gleichen Hydrolysebedingungen an 2′-, 5′- bzw. 3′-, 5′-Nucleosiddiphosphaten aus RNS erhielten. Durch Einwirkung von 5′-Nucleotidase wird aus

diesen Verbindungen ein Teil ihres Phosphates freigesetzt. Aus diesen Gründen kann man vermuten, dass es sich möglicherweise um zweifach veresterte Nucleotide handelt [vgl. (8)].

Die Bestimmungen der Spektra und des Ribosegehaltes dieser Verbindungen geben sie als Adenin-enthaltende Nucleotide zu erkennen. Es könnte daran gedacht werden, dass eine dieser Verbindungen u.U. ein durch Hydrolyse der Pyrophosphatbindung entstandenes Spaltprodukt des TPN darstellt, da in diesem Falle ein Mol Nicotinsäureamid-ribotid und 1 Mol Adenosin-2'-, 5'-diphosphat resultieren würden.

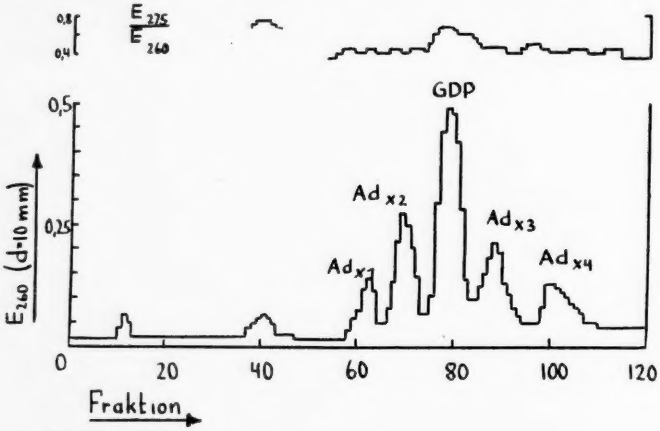


Abb. 29
Dowex—2, $HCOO^-$, 1.5×17.5 cm
Mischflasche: 450 ml H_2O
Reservoirflasche: 1—Ende 1 n $HCOONH_4$, 4 ml/Fraktion

Der weitere Verlauf des Leberchromatogramms hat bisher keine qualitativen Unterschiede gegenüber den freien Tumoren ergeben. Auf die quantitativen Unterschiede, besonders in Bezug auf die Mengen an vorhandenen Di- und Triphosphorsäureestern der Nucleoside wird noch besonders eingegangen werden (Tabellen 5—7).

b) Gehirn

Die Abbildung 30 stellt ein S—1-Chromatogramm des gesamten säurelöslichen Extraktes aus 7 g Kaninchenhirn dar. Die Ergebnisse entsprechen denen an Extrakten aus Rattengehirn (13). Als Vertreter der sogenannten ruhenden Gewebe ähnelt das Gehirn in seinem Gehalt an freien Nucleotiden am meisten den festen Tumo-

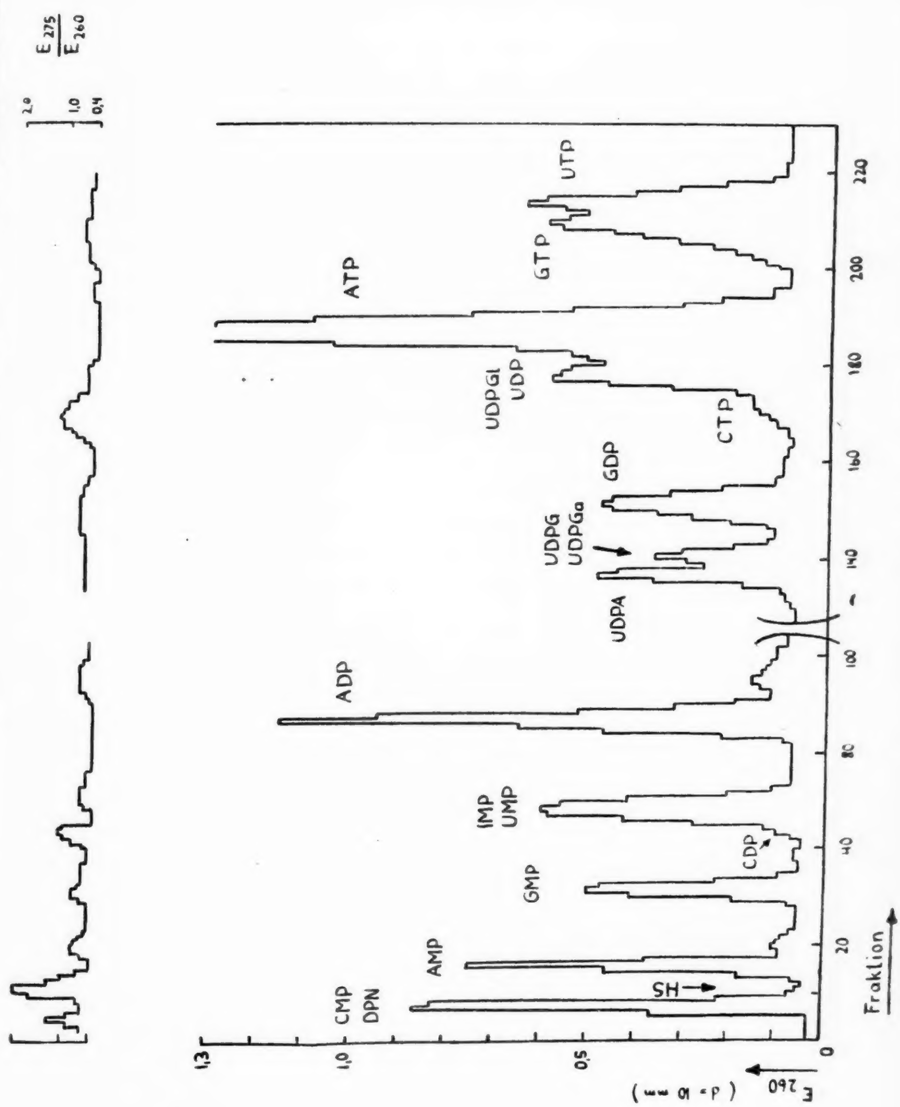


Abb. 30
Dowex—2, $HCOO^-$, 0.8 × 14.5 cm
Mischflasche: 325 ml H_2O

Reservoirflasche:	1— 28	1 n	HCOOH	
	29— 94	4 n	»	
	95—180	4 n	»	+ 0.4 n $HCOONH_4$
	181—Ende	4 n	»	+ 1.0 n »

4 ml/Fraktion

ren (vgl. Abb. 6 und 19). Fraktionen, die im Leberchromatogramm als Ad_x bezeichnete Verbindungen enthalten, wurden bisher in diesem Gewebe nicht gefunden. Ebenfalls konnten auch keine, für die Analyse ausreichenden Mengen an TPN isoliert werden. Unter Berücksichtigung der Schwierigkeiten einer schnellen Präparation dieses Gewebes — im Vergleich zu Tumoren und Leber — ist es nicht auszuschliessen, dass durch relativ langsameres Arbeiten ein Teil der Triphosphorsäureester der Nucleotide zu den entsprechenden Mono- und Diphosphaten abgebaut worden ist.

c) Muskel

In der Abbildung 31 ist ein Chromatogramm (S—1) des säurelöslichen Extraktes aus der quergestreiften Muskulatur der Maus dargestellt. Es ist besonders auffällig, dass fast alle Nucleotide als Triphosphate vorliegen. Abgesehen von IMP und den Mono- und Diphosphaten des Adenosins sind die Verbindungen CMP, CDP, GMP, GDP, UMP und UDP nicht in zur Analyse ausreichenden Mengen erhalten worden. Der relativ geringe Gehalt des Extraktes an den Monophosphorsäureestern von Adenosin und Inosin spricht für eine zeitlich gute Aufarbeitung des Gewebes, da bei länger dauernder Präparation grössere Mengen von ihnen auftreten. Weder in den früher (13) analysierten Extrakten aus Ratten- noch in den jetzt untersuchten Proben aus Kaninchen- und Mäusemuskulatur konnten UDP-derivate, wie sie so reichlich in anderen normalen und Tumorgeweben gefunden wurden, nachgewiesen werden.

d) Herzmuskel

Die Chromatographie des säurelöslichen Extraktes aus Kaninchenherz ist in der Abbildung 32 wiedergegeben. Die hohen Mengen an Adeninnucleotiden einerseits und die quantitativen Beziehungen zwischen energiearmen und energiereichen Formen der Nucleotide andererseits verleihen dem Chromatogramm eine sehr grosse Ähnlichkeit mit dem aus quergestreifter Muskulatur. Dieselben Verhältnisse wurden auch bei der Chromatographie der säurelöslichen Extrakte aus Rattenherz beobachtet (71). Im Gegensatz zur quergestreiften Muskulatur weist der Herzmuskel deutliche, wenn auch geringe Mengen an Mono-, Di- und Triphosphorsäureestern von

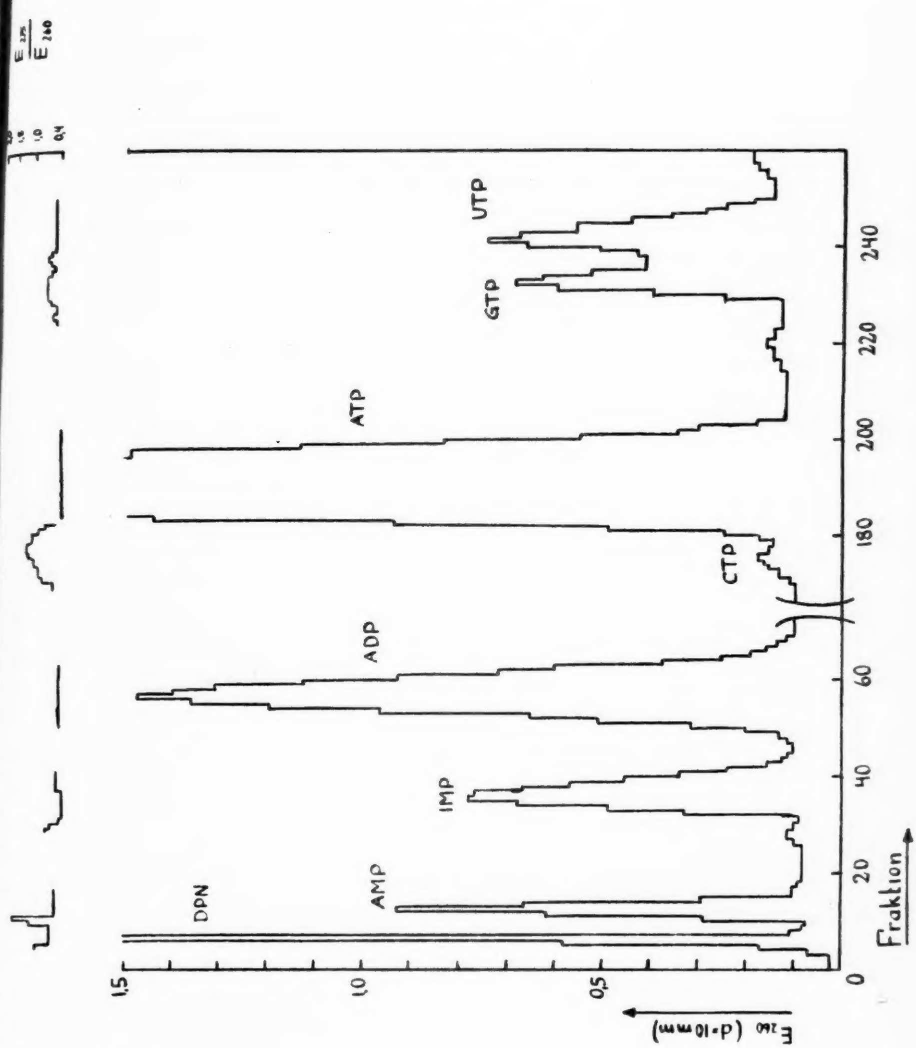


Abb. 31
Dowex—2, $HCOO^-$, 0.9×13.0 cm
Mischflasche: 330 ml H_2O
Reservoirflasche: 1— 80 4.0 n HCOOH
81—185 4.0 n » +0.3 n $HCOONH_4$
186—Ende 4.0 n » +1.0 »
4 ml/Fraktion

Guanosin, Cytidin und Uridin auf; ausserdem kommen im Herzmuskel auch einige UDP-derivate vor, die in der quergestreiften Muskulatur nicht gefunden wurden.

e) Niere

Die Chromatographie der säurelöslichen Extrakte aus Rattennieren ist an einem Beispiel in Abbildung 33 wiedergegeben. Ein Vergleich der UV-Absorptionsmaxima dieses Chromatogramms mit denen der Leber (vgl. Abb. 27) ergibt eine weitgehende Übereinstimmung hinsichtlich ihrer qualitativen Zusammensetzungen. Ein etwas grösserer Teil der freien Nucleotide liegt in der Niere als Di- und Triphosphorsäureester vor. Vergleichbare Untersuchungen an Nierengewebe von Mäusen und Kaninchen (71) ergaben eine gute Übereinstimmung mit den hier an der Rattenniere dargestellten Ergebnissen.

6.) TABELLARISCHE ZUSAMMENSTELLUNGEN DER ANALYSEN

In den Tabellen (2—4) sind die Ergebnisse der zur Identifizierung der freien Nucleotide aus der Mäuseleber (Abb. 27) durchgeführten Analysen zusammengefasst. Diese tabellarischen Übersichten dienen gleichzeitig als Beispiel für die Masstäbe, die für die Definierung der Zusammensetzungen an die anderen untersuchten Gewebeextrakte angelegt wurden.

Die Charakterisierung der 5′-Mono-, Di- und Triphosphorsäureester von Adenosin, Guanosin, Uridin und Cytidin beruht auf den Ergebnissen folgender Analysen, deren Durchführung im Abschnitt »Methoden» beschrieben wurde:

1) Chromatographie an S—1 und Rechromatographie an S—2.

2) UV-Absorptionsspektra im sauren und alkalischen Milieu (Tab. 2).

3) Gesamt- und säurelabiles Phosphat (Tab. 3).

4) Freisetzung von anorganischem Phosphat durch 5′-Nucleotidase aus Crotalus adamanteus (Tab. 3).

5) »Offensichtliche» Ribose (Tab. 4).

Die Ergebnisse der Ribosebestimmungen sind als »offensichtlich» bezeichnet, da die Purin als Base enthaltenden Nucleotide innerhalb

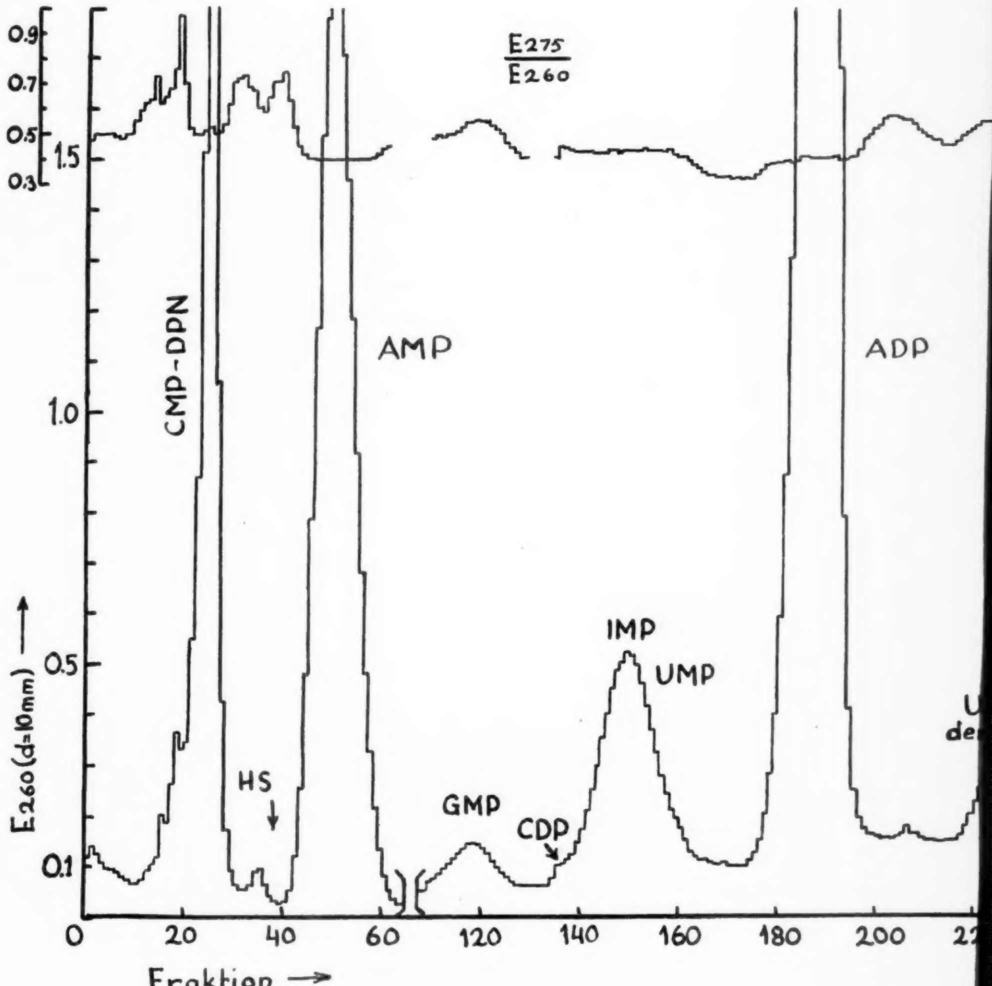
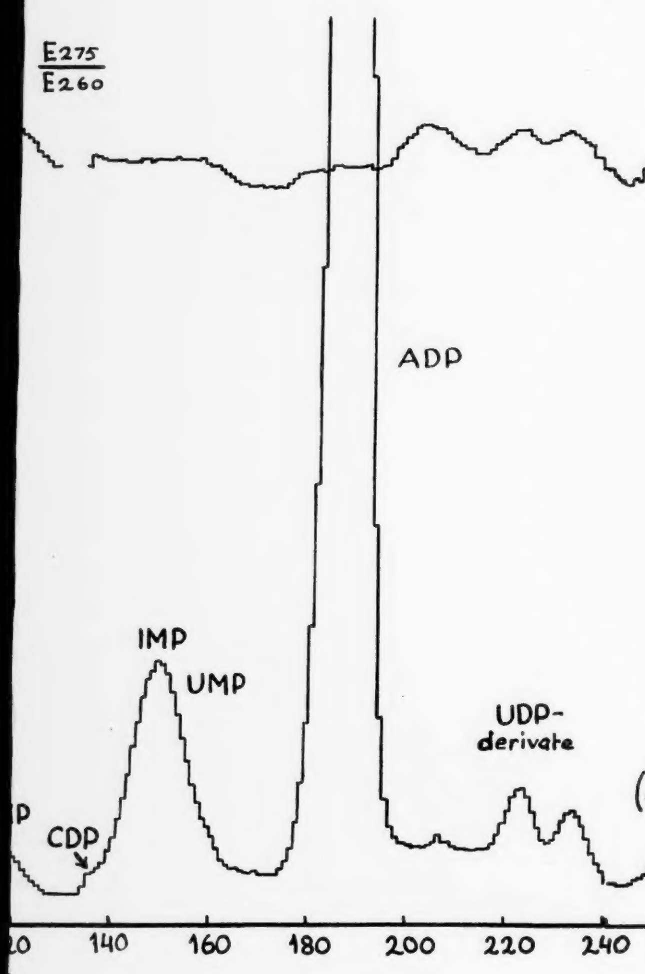
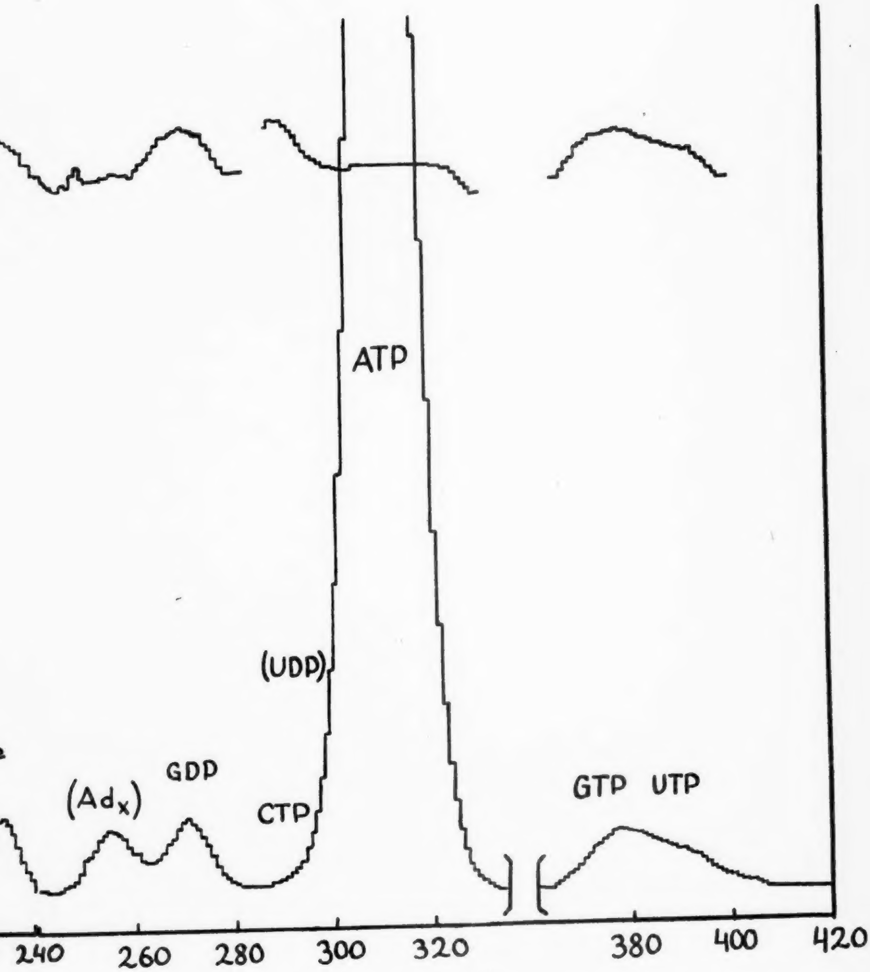


Abb. 32
Dowex—2, $HCOO^-$, 1.2×19.0 cm
Mischflasche: 510 ml H_2O
Reservoirflasche: 1— 60 1 n HCOOH
61—158 4 n »
159—283 4 n » +0.2 n $HCOONH_4$
284—Ende 4 n » +0.4 n $HCOONH_4$
4.1 ml/Fraktion



1.2 × 19.0 cm
H_2O
— 60 1 n HCOOH
—158 4 n »
—283 4 n » $+0.2$ n $HCOONH_4$
—Ende 4 n » $+0.4$ n $HCOONH_4$
ml/Fraktion



ATP
(UDP)
GDP
(Adx)
CTP
GTP UTP
240
260
280
300
320
380
400
420

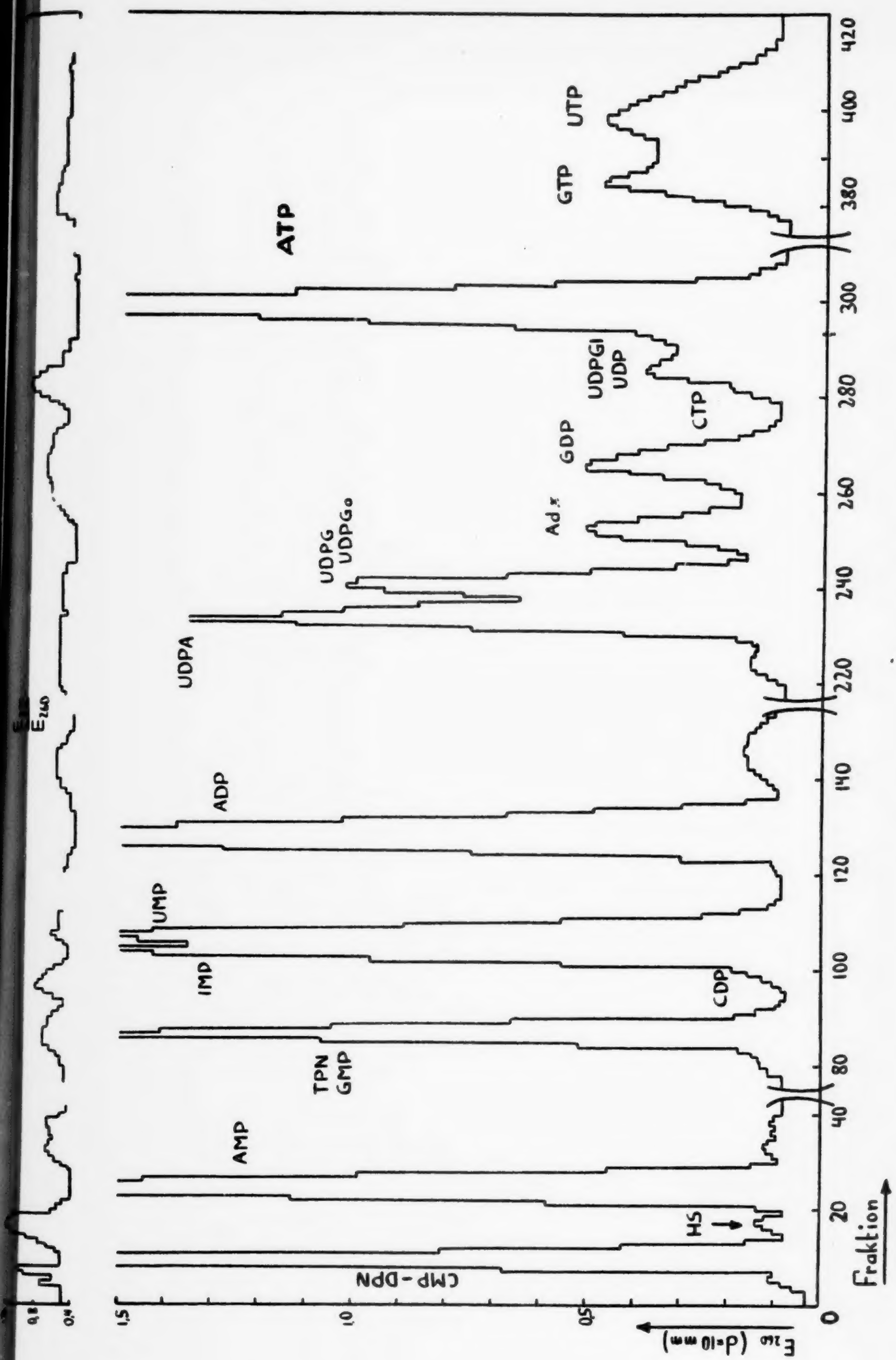


Abb. 33
Dowex—2, $HCOO^-$, 1.0×23.0 cm
Mischflasche: 500 ml H_2O
Reservoirflasche: 1—146 3 n HCOOH
147—281 4 n HCOOH+0.4 n $HCOONH_4$
282—Ende 4 n » +0.8 n » , 3 ml/Fraktion

von 15 min eine vollständige (1 M Ribose pro M Base), die Pyrimidinnucleotide aber nur eine teilweise (Uridin) oder überhaupt keine (Cytidin) Farbreaktion ergeben. Werden die Nucleotide unter den Versuchsbedingungen der Orcinreaktion hingegen länger als 15 min erhitzt, so beobachtet man im Falle der Purinnucleotide eine Abschwächung und bei den Pyrimidinnucleotiden eine Zunahme der Farbintensität. Das unterschiedliche Verhalten der Pyrimidin-

TABELLE 2

ABSORPTIONSSPEKTRA DER NUCLEOTIDE

Nucleotid	pH	Wellenlänge mμ		Optische Dichte bei einer gegeb. Wellenlänge / Optische Dichte des Maximums in Säure						
		Maximum	Minimum	Maximum	Minimum	230 mμ	245 mμ	260 mμ	275 mμ	290 mμ
CMP	H^+	280	241	1.00	0.12	0.26	0.13	0.50	0.97	0.74
	OH^-	273	250.5	0.70	0.48	0.62	0.50	0.56	0.67	0.20
CDP	H^+	280	241	1.00	0.12	0.26	0.13	0.50	0.97	0.74
	OH^-	273.5	251							
CTP	H^+	279.5	241	1.00	0.125	0.265	0.13	0.50	0.975	0.75
	OH^-	273	250							
UMP	H^+	261	230	1.00	0.22	0.22	0.55	0.99	0.615	0.03
	OH^-	260.5	241.5	0.78	0.55	0.74	0.57	0.77	0.45	0.03
UDP	H^+	261	230.5	1.00	0.22	0.225	0.55	0.99	0.615	0.03
	OH^-	261	241							
UTP	H^+	261	230	1.00	0.22	0.22	0.55	0.99	0.615	0.025
	OH^-	260.5	242							
GMP	H^+	256	228	1.00	0.22	0.24	0.735	0.96	0.69	0.45
	OH^-	265	231	0.93	0.35	0.36	0.65	0.93	0.80	0.11
GDP	H^+	256	228	1.00	0.225	0.24	0.735	0.96	0.69	0.45
	OH^-	265	231							
GTP	H^+	255.5	228.5	1.00	0.23	0.24	0.74	0.96	0.685	0.45
	OH^-	265	231							
AMP	H^+	257	228	1.00	0.21	0.24	0.63	0.98	0.40	0.04
	OH^-	260	230	1.04	0.24	0.24	0.60	1.04	0.38	0.02
ADP	H^+	257	228	1.00	0.21	0.24	0.63	0.98	0.40	0.03
	OH^-	260	230							
ATP	H^+	257	228	1.00	0.21	0.24	0.63	0.975	0.40	0.03
	OH^-	260	230							

Millimolare Extinktionskoeffizienten $\varepsilon^{H^+}_{\lambda\,max}$

Adeninnucleotide	14.4
Guaninnucleotide	13.3
Cytosinnucleotide	13.7
Urazilnucleotide	9.9

und Purinnucleotide ist durch die verschiedene Festigkeit der N-Glycosidbindung zu erklären.

6) UV-Absorptionsspektra der durch Hydrolyse erhaltenen Basen.

Die Charakterisierung der Uridin-5'-diphosphat-derivate basiert ausser auf den vorstehend genannten Kriteria (1—6) auf dem papierchromatographischen Verhalten der durch Hydrolyse abgespaltenen Hexosen oder Säure. Analysen wurden ausser an den isolierten auch an Verbindungen bekannter Zusammensetzung durchgeführt, soweit sie verfügbar waren. Falls dies nicht möglich war, wie z.B. bei UDP-derivaten, wurden die analytischen Ergeb-

TABELLE 3

PHOSPHATGEHALT DER NUCLEOTIDE

Nucleotid	M Gesamt-phosphat pro M Base	M säurelabiles Phosphat pro M Base	Freisetzung von M anorg. Phosphat durch 5'-Nucleotidase pro M Base*
CMP	0.96	0	0.99
CDP	2.12	1.05	0.98
CTP	3.05	2.00	1.00
UMP	1.00	0	1.02
UDP	1.93	1.00	1.01
UTP	2.92	1.95	0.97
UDPA	2.08	—	—
UDPG } **	2.04	—	—
UDPGa }			
UDPGl	1.95	—	—
GMP	1.03	0	0.97
GDP	2.00	1.02	0.96
GTP	2.95	2.03	0.975
GDPM	2.02	—	1.02
AMP	1.01	0	1.00
ADP	2.00	0.975	1.01
ATP	3.00	1.98	0.995
A2MP	1.00	0	0
A3MP	1.00	0	0

* Nach Umwandlung der Di- und Triphosphorsäureester in die entsprechenden Monophosphate.

** Konzentrationen beider UDP-derivate wie durch Rechromatographie an S—2 erhalten.

TABELLE 4

»OFFENSICHTLICHER« RIBOSEGEHALT DER NUCLEOTIDE

M PRO M BASE

Nucleotid	Dauer der Hydrolyse		
	15 min	60 min	120 min
CMP	0	0.02—0.03	0.04—0.055
CDP	0		
CTP	0		
UMP	0.16	0.4 —0.5	0.6 —0.67
UDP	0.15		
UTP	0.15		
*UDPA	0.14	0.4 —0.5	0.6 —0.67
*UDPG **	0.15		
*UDPGa **			
*UDPGl	0.16		
GMP	1.02	0.84—0.88	0.72—0.76
GDP	1.01		
GTP	1.05		
*GDPM	1.00		
AMP	1.00	0.83—0.87	0.70—0.74
ADP	0.97		
ATP	0.99		

* Bestimmt an den Nucleosidmonophosphorsäureestern.
** Vgl. Tab. 3.

nisse mit den in der Literatur beschriebenen Werten verglichen[1] (7, 8, 9, 22, 50, 85, 86).

In den Tabellen 5—7 sind die arithmetischen Mittelwerte der Konzentrationen an freien Nucleotiden in einigen Tumoren, ruhenden Geweben und Hefen aus jeweils 4—5 Bestimmungen zusammengestellt.

[1] Als Vergleichssubstanzen dienten — nach säulenchromatographischer Überprüfung bzw. Reinigung — Proben, die von *Dr. W. E. Cohn*, Oak Ridge National Laboratories, Oak Ridge, Tenn., USA und den *Pabst-Laboratories*, Milwaukee, Wisc., USA, grosszügigerweise zur Verfügung gestellt wurden sowie von der Fa. *Sigma Chemical* Co., St. Louis, Mo., USA, gelieferte Präparate.

TABELLE 5

FREIE NUCLEOTIDE IN TUMOREN (μM/100 G FEUCHT)

Nucleotid	I	II	III	IV	V	VI
AMP	40	32	44	37	41	42
ADP	60	61	89	72	80	71
ATP	71	78	91	91	95	86
GMP	7	5	8	7	4	5
GDP	11	14	17	15	7	10
GTP	20	24	15	12	8	17
UMP	7	8	12	7	6	7
UDP	13	10	10	9	3	8
UTP	17	23	17	16	11	14
CMP	6	5	8	7	4	6
CDP	1	2	2	1	0.6	1
CTP	12	15	13	16	8	9

I: Flexner-Jobling-Carcinom, II: Walker-256-Carcino-Sarkom, III: Yoshida-Sarkom, IV: Sarkom-37 (Ascitesform), V: Yoshida-Sarkom (Ascitesform), VI Ehrlich (diploid) (Ascitesform)

TABELLE 6

FREIE NUCLEOTIDE IN RUHENDEN GEWEBEN (μM/100 G FEUCHT)

Nucleotid	I	II	III	IV	V	VI
AMP	85	92	60	9	4	42
ADP	111	74	75	40	20	127
ATP	45	31	75	350	320	253
GMP	17	15	19			
GDP	15	12	14			
GTP	9	7	11	8	7	5
UMP	17	14	22			
UDP	13	14	13			
UTP	8	7	12	7	7	5
CMP	8	9	7			
CDP	2	2	1			
CTP	3	2	4	1	1.5	0.9

I: Leber (Maus), II: Leber (Ratte), III: Niere (Ratte), IV: Muskel (Ratte[1]) V: Muskel (Maus[1]), VI: Herz (Ratte)

[1] Nach Evipannarkose (vgl. Text)

TABELLE 7

FREIE NUCLEOTIDE IN HEFEN (μM/100 G FEUCHT)

Nucleotid	I	II	III	IV
AMP	25	41	41	62
ADP	39	52	39	73
ATP	80	76	94	80
GMP	14	12	8	21
GDP	22	8	17	13
GTP	19	29	24	21
UMP	10	11	15	20
UDP	9	22	17	14
UTP	21	16	27	22
CMP	7	11	13	10
CDP	3	1	2	2
CTP	12	16	8	14

I: Hefe von Dr. F. Hillringhaus
II: Löwenhefe, München
III: Sandvosshefe, München
IV: Bopp-Beyerhefe, Marburg/Lahn

C DISKUSSION DER ERGEBNISSE

Wie einleitend dargelegt, ist das Vorkommen der 5'-Mono-, Di- und Triphosphorsäureester von Adenosin seit vielen Jahren bekannt, das der analogen Ester von Guanosin, Cytidin und Uridin aber erst seit neuester Zeit. Auch aus sauren oder alkalischen Hydrolysaten der Nucleinsäuren konnten bisher keine 5'-Mononucleotide isoliert werden. Erst Cohn und Volkin (70) haben gezeigt, dass durch Einwirkung einer gereinigten Phosphodiesterase auf Ribonucleinsäuren Nucleosid-5'-phosphate entstehen.

Die in dieser Arbeit besprochenen 5'-Mono-, Di- und Triphosphorsäureester von Adenosin, Guanosin, Uridin und Cytidin sind aber durchaus eigenständige Verbindungen und können nicht als Spaltprodukte der Nucleinsäuren, freigesetzt durch die Extraktion des Gewebes mit verdünnter Perchlorsäure in der Kälte, aufgefasst werden. Diese Möglichkeit kann deshalb ausseracht gelassen werden, da weder durch saure noch durch alkalische Hydrolyse aus den Nucleinsäuren Pyrophosphorsäureester der Nucleoside entstehen können. Die Eigenständigkeit der freien Nucleotide wurde auch experimentell untersucht und bestätigt. In bereits zitierten Untersuchungen (vgl. Einleitung) wurde nämlich gefunden, dass zu verschiedenen Zeitpunkten nach Verabreichung von C^{14}-markierten Verbindungen und von markiertem anorganischen Phosphat (P^{32}) die freien und polymerisierten Nucleotide völlig verschiedene spezifische Aktivitäten aufweisen [LePage (32), LePage und Edmonds (33), Hurlbert (15), Hurlbert und Potter (16, 17), Schmitz und Mitarbeiter (10—13) sowie Schmitz und Saukkonen (38, 71)].

Es wurde versucht, die in der Einleitung gestellte Frage nach möglichen Unterschieden in den Zusammensetzungen der Reservoirs an freien Nucleotiden von ruhenden und wachsenden Geweben vor allem von der qualitativen und — soweit möglich — auch von der quantitativen Seite her zu beantworten. Die Ergebnisse zeigen,

dass mit Ausnahme der Blutzellen (87), alle bisher untersuchten Gewebe neben den 5'-Mono-, Di- und Triphosphaten von Adenosin beachtliche Mengen analoger Ester von Guanosin, Cytidin und Uridin enthalten. Ausser in den hier beschriebenen Geweben, wurden die neuen Nucleotide und einige ihrer Derivate auch in den säurelöslichen Extrakten von Thymus, Lunge, Milz, Pankreas, Testes (71) und Milchdrüse (60) gefunden. Strominger (68) isolierte aus den säurelöslichen Extrakten des Hühnereileiters vier UDP-N-acetylaminozucker, die er — ohne nähere Definition ihrer chemischen Unterschiede — auf der Basis ihrer Reihenfolge bei der Eluierung an Dowex-1 als $UDPA_1$, $UDPA_2$, $UDPA_3$ und XA_4 bezeichnete; aus dem gleichen Gewebe isolierte Strominger Guanosin-5'-pyrophosphat-mannose, während er in der Hühnerleber nur $UDPA_1$ fand.

Im Hinblick auf den freien Nucleotidgehalt unterscheiden sich — unter gleichen Versuchsbedingungen — die qualitativen und quantitativen Zusammensetzungen der säurelöslichen Extrakte eines bestimmten Gewebes von verschiedenen Tieren nur geringfügig. So wurde z.B. gefunden, dass die Konzentrationen an freien Nucleotiden in säurelöslichen Extrakten aus Hirn von Ratten, Hunden, Mäusen, Meerschweinchen und Kaninchen, aus Lebergewebe von Ratten, Mäusen, Meerschweinchen und Kaninchen, sowie aus Blutzellen von Mäusen, Ratten, Hunden und Meerschweinchen nur unwesentliche Unterschiede aufweisen (71).

In Untersuchungen an quergestreifter Muskulatur hingegen wurden zunächst unterschiedliche Konzentrationen zwischen Mäusen einerseits sowie Ratten und Meerschweinchen andererseits beobachtet: Erstere enthielten etwa 20% weniger ATP als die letzteren, während die Konzentrationen an AMP, ADP und IMP in etwa gleicher Grössenordnung erhöht waren. Es wurde vermutet, dass diese Unterschiede durch methodische Fehler bedingt sind, da von den sich wesentlich ruhiger verhaltenden Ratten und Meerschweinchen das Muskelgewebe unter günstigeren Voraussetzungen als von den sehr unruhigen Mäusen präpariert werden kann. Es wurden daher säurelösliche Gewebeextrakte aus der quergestreiften Muskulatur von Mäusen, Ratten und Meerschweinchen, die zuvor mit Evipan[1] narkotisiert waren, chromatographiert und dabei ge-

[1] Natriumsalz der N-Methyl-cyclohexenylmethylbarbitursäure (Hersteller: Farbenfabriken Bayer A. G., Wuppertal-Elberfeld).

funden, dass unter diesen Bedingungen alle drei Tierarten gleiche Konzentrationen an freien Nucleotiden enthalten (71).

In Versuchen an der quergestreiften Muskulatur von Ratten, die durch Injektion von $MgSO_4$ narkotisiert waren [1 ml einer 4%igen Lösung pro Kilogramm Körpergewicht (73)], wurden Unterschiede gegenüber nicht oder durch Evipan narkotisierten Tieren beobachtet: Durch Ionenaustauschchromatographie konnten bei durch $MgSO_4$ narkotisierten Tieren nur noch Spuren von AMP, IMP und geringe Mengen von ADP unter gleichzeitiger Erhöhung des ATP-Gehaltes isoliert werden (71). Dies dürfte auf einer Hemmung der Muskel-Adenosintriphosphatase durch Mg^{++} beruhen, da in Chromatogrammen aus der Leber dieser Tiere keine gleichsinnigen Veränderungen beobachtet werden konnten. Dieser Befund wird verständlich unter Berücksichtigung der Hemmbarkeit der Adenosintriphosphatase des Muskels — im Gegensatz zum gleichen Ferment der Leber — durch Mg^{++}.

Das Vorkommen der neuen Nucleotide konnte inzwischen auch in einigen Bakterien festgestellt werden. Strominger isolierte aus dem säurelöslichen Extrakt von mit Penicillin vorbehandelten Stämmen von *Staphylococcus aureus* Uridin-5'-diphosphat-derivate und vermutete ausserdem das Vorkommen von Cytidin-5'-monophosphat (74). In diesem Zusammenhang müssen auch die Arbeiten von Park (75, 76, 77) erwähnt werden, dem es gelang, aus *Staphylococcus aureus* neben UDP-N-acetylaminozuckern auch UDP-aminosäure-derivate zu isolieren. Die von Kita und Peterson (65) sowie von Ballio und Casinovi (78) vorgelegten Untersuchungen an *Penicillium chrysogenum* unterstützen ebenfalls die Annahme eines ubiquitären Vorkommens von Nucleosid-5'-mono- und polyphosphorsäureestern, da letztgenannte Autoren in den Extrakten aus diesem Bakterium neben den Coenzymen DPN und TPN sowie einer Reihe noch nicht identifizierter Nucleotide, CMP, IMP, AMP, ADP, ATP, UMP, UDP-derivate, UTP, GMP, GDPM und GTP nachweisen konnten [vgl. die erwähnten Veröffentlichungen aus dem Arbeitskreis von Leloir (7, 56, 57, 58, 59)].

Unter Einschluss der in dieser Arbeit vorgelegten Ergebnisse lassen sich bisher keine grundlegenden qualitativen Unterschiede zwischen den in ruhenden und wachsenden Geweben vorkommenden Nucleotiden erkennen. Die in fast allen ruhenden Geweben gefundenen Adenosin-phosphat-derivate (Ad_x, vgl. Abb. 29)

konnten zwar bisher nicht aus Tumoren, wohl aber aus wachsenden Hefen isoliert werden. Über die Bedeutung dieser Verbindungen ist nichts bekannt und auch die bisherigen Untersuchungen lassen nur Vermutungen über ihre Entstehung bzw. Herkunft zu. Die nach Dutton und Storey (61, 92) an der Glucuronidsynthese als Coenzym beteiligte Uridin-5'-diphosphat-glucuronsäure konnte bisher nicht sicher in Ascitestumoren und im festen Yoshida-Sarkom nachgewiesen werden, obwohl andere feste Tumoren, wenn auch unterschiedliche, so doch der Leber vergleichbare Konzentrationen enthielten. Bei der Darstellung der Ergebnisse wurde bereits bemerkt, dass die Möglichkeit des Vorhandenseins von UDPGl im Yoshida-Sarkom durchaus besteht, da durch Rechromatographie an S—2 Spuren eines Uracilnucleotids gefunden wurden, dessen Position bei der Eluierung an diesem System der dieses Coenzyms entspricht.

In den Tabellen 5—7 ist eine Übersicht über die Konzentrationen der freien Nucleotide in einigen ruhenden und wachsenden Geweben zusammengestellt. Die dort aufgeführten Werte dürften wohl nur hinsichtlich der Gesamtmengen eines Nucleotides mit einer bestimmten Base Gültigkeit besitzen. Die Beurteilung der quantitativen Verhältnisse zwischen energiearmen und energiereichen Formen eines Nucleotids erfordert aus folgenden Gründen grösste Zurückhaltung:

1) Berücksichtigung der leichten oder schweren Präparation eines Gewebes und der dadurch bedingten unterschiedlichen Zeit, die von der Tötung bis zur Einfrierung des Gewebes in flüssiger Luft vergeht.

2) Möglichkeit einer schnelleren Spaltung von energiereichen Phosphatbindungen in einzelnen Geweben entsprechend ihrer Fermentausstattung.

3) Einwirkung von Säuren während der Extraktion und der Chromatographie. Während der Gewebeextraktion durch verdünnte Perchlorsäure bei 0 bis $+2°$ nur eine sehr geringe Bedeutung hinsichtlich der Hydrolyse von energiereichen Phosphatbindungen zukommen dürfte, sind den Einwirkungen der zur Eluierung der Anionenaustauscher bei Zimmertemperatur verwendeten Säurekonzentrationen und den damit notwendigerweise verknüpften längeren Aufenthalten der Verbindungen in der Säure, wesentlich grössere Bedeutung zuzumessen.

Die Abhängigkeit der relativen Konzentrationen an Nucleosid-mono- und pyrophosphorsäureestern von der Isolierungsmethode geht aus einem Vergleich der hier mitgeteilten mit den von LePage (72) durch fraktionierte »Bariumfällung» gefundenen Werten hervor.

LePage führte seine Untersuchungen 1947—1948 durch, zu einer Zeit also, in der ausser einigen Cofermenten nur AMP, ADP und ATP als freie Nucleotide bekannt waren. Dies mag erklären, dass die von LePage gemessenen Konzentrationen an Adeninnucleotiden insgesamt etwas höher als die hier mitgeteilten liegen. Hinsichtlich der Relationen von energiereichen und energiearmen Nucleotiden in ruhenden und wachsenden Geweben besteht zwischen den Ergebnissen von LePage und denen, die in Tabelle 5 angegeben sind, eine grundsätzliche Übereinstimmung in dem Überwiegen der energiereichen über die energiearmen Nucleotide bei schnellwachsenden Tumoren.

Ebenso wie LePage durch »Bariumfraktionierung», konnte hier durch Chromatographie gezeigt werden, dass der Muskel unter den normalen Geweben insofern eine Sonderstellung einnimmt, als bei ihm die freien Nucleotide fast nur in der energiereichen Form vorkommen (vgl. Abb. 31). Dieser Befund drängt einen Vergleich zwischen Muskel- und Tumorgewebe auf. Die Ähnlichkeit zwischen beiden Geweben entspricht teleologisch vielleicht dem ungeheuer grossen Energiebedarf des Tumors für seine synthetischen Leistungen im Rahmen des Stoffwechsels und des Wachstums; und in diesem Sinne ist auch der Tumor ein »schwer arbeitendes Gewebe» wie der Muskel.

Zusammenfassend kann man feststellen, dass bisher keine Anhaltspunkte für kennzeichnende Unterschiede zwischen ruhenden und wachsenden Geweben im Hinblick auf die qualitative Zusammensetzung ihrer Reservoirs an freien Nucleotiden bestehen, während quantitative Verschiedenheiten erkennbar sind.

D ZUSAMMENFASSUNG

Die vorliegende Arbeit stellt eine vergleichende Untersuchung über das Vorkommen von freien Nucleotiden in ruhenden und wachsenden Geweben dar. Es wurden folgende Gewebe untersucht:

1) *Feste Tumoren:* Yoshida-Sarkom, Jensen-Sarkom, Walker-256-Carcino-Sarkom, Flexner-Jobling-Carcinom.

2) *Ascitestumoren* (Zellen und Sera getrennt): Yoshida, Sarkom-37, Ehrlich (diploid), Ehrlich-Colchicin-resistent und Ehrlich-»Wärmestamm«.

3) *Hefezellen:* Bier- und Bäckerhefen.

4) *Ruhende Gewebe:* Leber, Gehirn, Muskel (quergestreift), Herzmuskel und Niere.

Der qualitative und soweit möglich auch quantitative Vergleich der Verteilungsmuster der freien Nucleotide in wachsenden und ruhenden Geweben ergab:

a) Die weite Verbreitung der erst kürzlich entdeckten 5′-Mono-, Di- und Triphosphorsäureester von Guanosin, Cytidin und Uridin (sowie Uridin-5′-diphosphatderivaten) neben den analogen Phosphorsäureestern des Adenosins.

b) Bei den Tumoren liegt das Verhältnis der Mono- zu den Di- und Triphosphorsäureestern der Nucleoside zugunsten der energiereichen Formen.

c) Im Gegensatz zu den festen Tumoren enthalten die Ascitestumoren nur geringe Mengen an Uridin-5′-N-acetylglucosamin und Uridin-5′-diphosphat-glucose bzw.-galactose.

d) Soweit in ruhenden Geweben Uridin-5′-diphosphat-N-acetylglucosamin und Uridin-5′-diphosphat-glucose bzw. Uridin-5′-diphosphat-galactose vorkommen, enthalten diese etwa gleiche oder bis zu 30% geringere Mengen an Uridin-5′-diphosphat-N-acetylglucosamin als an den beiden anderen Uridin-5′-diphosphat-deriva-

ten. Die festen Tumoren hingegen enthalten nach den bisherigen Ergebnissen bis zu 60% mehr Uridin-5'-diphosphat-N-acetylglucosamin als Uridin-5'-diphosphatglucose bzw. Uridin-5'-diphosphat-galactose.

e) Die in Leber, Niere und einigen anderen ruhenden Geweben sowie in wachsenden Hefen gefundenen Adenosin-phosphatderivate (Ad_x), welche weder mit den 5'-Mono-, Di- und Triphosphorsäureestern des Adenosins noch dessen 2'- bzw. 3'-Monophosphorsäureestern identisch sind, konnten bisher in keinem Tumor nachgewiesen werden.

f) Aus dem säurelöslichen Extrakt von Hefezellen verschiedener Herkunft wurden durch Chromatographie neue Verbindungen abgetrennt, bei denen es sich auf Grund der bisherigen Befunde sehr wahrscheinlich um Nucleotide handelt. Ihre Zusammensetzung ist noch nicht endgültig gesichert.

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EFFECTS OF THE FINNISH SAUNA ON CIRCULATION

STUDIES ON HEALTHY AND HYPERTENSIVE SUBJECTS

BY

A. EISALO

HELSINKI 1956

HELSINKI 1956
Mercatorin Kirjapaino

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A. E.

CONTENTS

REVIEW OF THE LITERATURE

The Sauna[1] has been used by the Finns for centuries. They take a sauna at least once a week, and there are few things that can prevent them from doing so. Räsänen (1951) reports that out of 111 patients with coronary thrombosis 107 used the sauna regularly after recovery. Nor is age a restriction; the sauna is used by infants as well as elderly people.

EFFECT OF AMBIENT HOT AIR UPON THE CIRCULATION

The circulation plays an important part in regulating the balance of heat in the body. When the body temperature rises and the organism tries to augment its loss of heat, the excess heat from the deeper tissues is conveyed to the lungs and the surface of the skin through the circulation. The vessels of the skin dilate and the dermal circulation increases.

If the environmental temperature exceeds that of the body, heat is transferred to the body by radiation, conduction and convection. When the dermal circulation is increased, heat is transferred to the body from the environment and the body temperature starts to rise proportionally to the circulation. A decrease in the cutaneous blood flow would under these circumstances retard the rise in body temperature. This, however, does not occur when the external temperature exceeds that of the body and the only available method for losing heat is transpiration. The rate of transpiration depends not only upon the amount of sweat excreted but also upon the humidity of the external air.

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[1] Sauna = a hot steam bath taken in an enclosed room heated by a stove. A little water is thrown onto stones on top of the stove to raise the temperature temporarily.

When the peripheral blood volume increases, a corresponding decrease has been observed in the deeper parts of the body, especially in the splanchnic region (Müller 1905, Rein 1931). Sjöstrand (1935) observed in his experiments that the lungs of animals chilled down before killing weighed more than those of animals kept in a warm environment. This, he thought, proved that the lungs contain less blood in a warm environment than in a cold. Glaser (1949) reported that the vital capacity decreased by 200—480 ml. in an environmental temperature below +14°C and when his experimental subjects were kept 2 hours at +40.5°C the vital capacity increased by 180—540 ml. He explained the change in the vital capacity as a sign of the decreased blood volume in the lungs due to the shift of blood into the peripheral tissues. Using X-ray methods Glaser et al. (1950) observed a decrease in the size of the liver and of the pulmonary vessels in a warm environment; this, they thought, was caused by the transfer of blood to the periphery.

Changes in the Cardiac Output. — Determinations have been made of the cardiac output of experimental animals and of human subjects in a hot environment. Uyeno (1923) determined the cardiac output in anaesthetized cats using Fick's method. When the body temperature of the cats had increased to +39°C, there was an increase of 20—30% in the cardiac output compared with the starting value. The cardiac output values varied greatly in the experiments made by Brenning (1938) on anaesthetized rabbits. The determinations were made by Fick's method. In the first group of experiments a decrease in the cardiac output was observed at +39.7°C, and the cardiac output continued to decrease with increasing temperature. In the second group with corresponding temperatures (rectal temperature 39.3°C and 41.9°C) a slight increase was first observed in the cardiac output and then a decrease. Daily and Harrison (1948) demonstrated an increase in the cardiac output of anaesthetized dogs with a maximum increase of 60% at body temperatures ranging between +42°C and +44°C. When the temperature rose above these values the cardiac output started to fall. The fall was preceded by a decrease in the blood pressure. Prec et al. (1949) reported that the cardiac output of anaesthetized dogs decreased in a warm environment; this decrease was presumably due to a decreased return of venous blood.

Barcroft and Marshall jr. (1923) reported an increase of the cardiac output in human subjects in a hot environment. The mean increase was 3—4 l. after one hour in an ambient temperature of 40°C. The cardiac output was determined according to the method of Redfield, Bock and Meakins, which is based on the amount of oxygen and carbon dioxide in mixed venous blood as determined from the gas tensions of expired air and on the dissociation curves of these gases in blood, combined with a measurement of the total O_2 consumption. Marschak (1929) observed an increase in the cardiac output of three experimental subjects at an ambient temperature of +46°C and a relative humidity of 50%. After one hour in this environment the increase in the cardiac output was ½—1 l. The determination of the cardiac output in these experiments was based upon the use of ethyl iodide fumes. Grollman (1930) used his own acetylene method in measurements of the cardiac output. In reviewing literature on this subject he criticised the previous methods used for determining cardiac output because of their inaccuracy. In his own investigations he reported an increase of 1.3 l. at an ambient temperature of +45°C. Ude (1932) observed an increase in the cardiac output of up to 138% in 20—30 min., when his subjects were kept at an ambient temperature between +60° and +70°C. The determinations were made by the acetylene method. Eismeyer and Czyrnick (1934) performed experiments using Grollman's acetylene method. The subjects sat for 10 min. in a steam bath at +46°C. The cardiac output rose by 60%. Asmussen (1940) believed that the cardiac output decreases at first in a warm room, due to the transfer of blood to the periphery and a corresponding decrease in the venous return, but starts to increase after some time. Scott et al. (1940) determined the cardiac output at an ambient temperature of +32°C using the acetylene method as well as Bazett's method, by which the cardiac output is determined from the blood pressure and the speed of the pulse wave. The values for the cardiac output were at first above normal when the subjects remained in a recumbent position, but decreased gradually to normal or sub-normal values. When the subjects stood in a warm environment, the values were usually slightly below the starting value. Wezler and Thauer (1943) determined the cardiac output in recumbent subjects at an ambient temperature of +50°C according to Wezler's and Böger's methods. At a body

temperature of +40°C the cardiac output was 125% higher than at +37°C. These investigators observed considerable individual differences in the cardiac output in an environment of +50°C. In one subject the cardiac output was observed to be 9.5 l., in another 17.5 l., under the same conditions. Blockley and Taylor (1948) observed that the cardiac output of healthy recumbent subjects increased 2—5 times in 20 min. at an ambient temperature ranging between +37.5°C and 121°C.[1] The cardiac output was determined by Liljestrand and Zander's method.

Changes in the Stroke Volume. — Wiggers and Orias (1932) reported that the stroke volume of anaesthetized dogs decreased continuously with increasing body temperature. When the temperature reached +44°C, the stroke volume had decreased from 135 ml. to 60 ml.

Grollman (1930) found no change in the stroke volume at the ambient temperature of +45°C. Eismeyer and Czyrnick (1934) reported a decrease in the stroke volume in a steam bath at +46°C and an increase in a water bath at the same temperature. Wezler and Thauer (1934) observed an increase in the stroke volume of up to 140—150 cm^3 at an ambient temperature of +50°C.

Changes in the Circulation Time. — The circulation time decreases in a warm environment. Kissin and Bierman (1933) reported a decrease from 20 sec. to 9 sec. with an increase in the rectal temperature to 39.4°C—40.4°C. They determined the circulation time by the intravenous injection of sodium cyanide into 6 subjects. Hines and Kvale (1944) reported a decrease of 1 sec. in the circulation time from the hand to the tongue and a decrease of 3 sec. from one hand to the other, while the skin temperature increased from +30.3°C to +34°C on the fingers and from +32°C to +36°C on the toes.

Changes in the Pulse Rate. — One of the most general signs of the effect of a warm environment upon the circulation is the increase in the pulse rate. Grollman (1930) measured the pulse rate in healthy subjects at increasing temperatures. At an environmental temperature between +20°C and +30°C the pulse rate increased by 6 beats per min., between +30°C and +40°C, by c. 9 beats per min. and between +40°C and +45°C by c. 11 beats per min.

[1] Temperatures given in Fahrenheit in the literature have been converted to the centigrade scale.

Wezler and Thauer (1943) reported a maximum pulse of 145/min. in healthy subjects after 3 hours at + 50°C.

Changes in Blood Pressure. — Cheer (1928) observed in his experiments on anaesthetized dogs that in a warm environment neither the systolic nor the diastolic blood pressures changed until the body temperature of the dogs reached +40°C, at which point both pressures started to decrease. Wiggers and Orias (1932) reported an increase in both the systolic and the diastolic blood pressures of anaesthetized dogs in a warm environment. At a body temperature of +44°C the systolic blood pressure was 70 mmHg and the diastolic 30 mmHg higher than the starting values. As the body temperature rose above +44°C, a sharp fall in both blood pressures was observed. Brenning (1938) observed a slight increase in the systolic blood pressure of anaesthetized cats kept in a warm environment. According to Proskauer *et al.* (1945) the systolic blood pressure increased by 10—80 mmHg within 6—10 min. after the beginning of the heating. When the heating was slow, an increase of 23—106 mmHg in 16—24 minutes was observed in the systolic blood pressure. In hypertensive rats the systolic pressure also rose and stayed high for up to 2 hours when the animals were kept continuously in a warm environment. Rodbard and Feldman (1946) and Rodbard et al. (1949, 1950) observed an increase of 3—19 mmHg in the systolic and 1—12 mmHg in the diastolic pressures of turtles when heated. Daily and Harrison (1948) did not observe any change in the mean arterial blood pressure of dogs before the body temperature had reached +42°C, at which temperature the pressure started to decrease. Prec *et al.* (1949) determined the mean arterial blood pressure at the femoral artery in anaesthetized dogs, and increased the body temperature of the dogs up to +42°C. In 4 animals out of 9 a decrease in pressure was observed, in 3 dogs an initial increase was followed by a fall, the maximum decrease being 57% measured from the starting value. Olmstedt *et al.* (1951) determined the mean arterial blood pressure in rats and observed an increase of 42 mmHg in the animals whose body temperature was increased to +45°C in 10 minutes. In those animals whose temperature was increased rapidly (3 min.) the mean arterial blood pressure increased by 20 mmHg. If the starting value for the mean arterial blood pressure was below 120 mmHg the increase was 49% of the starting value, whereas if the starting value was over 140

mmHg the increase in the mean pressure due to the increasing heat was only 25% of the starting value. Ederström (1954) observed an increase of c. 20 mmHg in dogs when the body temperature had risen to +40°C.

The results of experiments on the circulation of animals in a warm environment are not directly comparable with the results obtained under the same conditions with human subjects. The animals are anaesthetized, and the differences in the distribution of the sweat glands and in the respiration rate in a warm environment may influence the final results (Grollman 1930).

Sayers and Harrington (1921, 1923), in their investigations into the effect of a warm environment upon the blood pressure of human subjects, reported a fall of c. 20 mmHg in the systolic blood pressure and a fall of 6—20 mmHg in the diastolic pressure. This decrease in the blood pressure was obtained by keeping healthy subjects sitting at an ambient temperature of +33.5°C—+37.8°C and a relative humidity of c. 90%. McConnell and Houghton (1923) and McConnell, Houghton and Phillips (1923) observed a rise in the systolic and a fall in the diastolic pressure, when healthy subjects were kept in a hot room. Adolph and Fulton (1924) measured the systolic and the diastolic blood pressures of healthy subjects at an ambient temperature of +39.5°C—+40.7°C. A rise of c. 10 mmHg was observed in the systolic blood pressure whereas the diastolic pressure fell to values which were impossible to determine by the indirect method of measuring. Most of the subjects sat during the experiment. In addition, the authors performed blood pressure measurements on five subjects in standing, sitting or recumbent positions. The results were similar, but it was evident that the recumbent subjects could stand the heat longer than the others. McConnell and Yagloglou (1925) reported an increase in the systolic and a decrease in the diastolic pressure in a hot room. According to Bazett (1927, 1931, 1938) there is no change in the systolic blood pressure in mild heat, but in a hot environment a decrease in both the systolic and the diastolic pressures will be observed. The behaviour of the blood pressure in warm and hot environments is dependent upon the contraction and dilatation of the blood vessels, upon the magnitude of the cardiac output and upon the changes in the pulse rate. If the blood pressure rises considerably in a hot environment, it is, according to Bazett, a sign of contraction in the

arteries and hence, a general warning signal of the organism. Grollman (1930) reported experiments in which the subjects were kept in a recumbent position at the ambient temperature of +45°C. No change in either the systolic or the diastolic pressure was observed. Gottlieb (1935) reported no increase in the systolic blood pressure of healthy subjects kept in a recumbent position at an ambient temperature increasing from +23.6°C to +33.2°C, whereas the diastolic pressure rose on the average by 3.0 mmHg. Scott *et al.* (1940) reported that when recumbent healthy subjects were exposed to an ambient temperature of +32°C, the diastolic blood pressure first rose by c. 5 mmHg or fell slightly, and later the pressure levelled off to values close to the starting value. If the subjects stood in the warm environment, the diastolic pressure first showed a rise and then fell to about 7 mmHg below the starting value. Scott *et al.* could not detect any changes in the systolic blood pressure under the same conditions. Hyndman and Wolkin (1941) reported a fall of c. 10 mmHg in both the systolic and the diastolic blood pressures of sympathectomized subjects with normal blood pressure, when they were kept in a recumbent position for one hour at an ambient temperature of +45°C—+48°C. In hypertensive subjects the systolic pressure fell c. 30 mmHg and the diastolic c. 20 mmHg under the same conditions. Wezler and Thauer (1943) observed a fairly sharp fall in the diastolic blood pressure in a hot environment. At a body temperature of +40°C a fall of 30 mmHg was observed. The subjects were in a recumbent position during these experiments. Glickman *et al.* (1947) observed in 5 subjects a mean rise of 6.4 mmHg in the systolic pressure during the first two minutes in a hot room and a simultaneous decrease of 3.1 mmHg in the diastolic pressure. The subjects remained sitting for one hour in a hot environment. This slight increase in the systolic pressure was followed by a fall, and 12—15 min. after the beginning of the experiment the systolic blood pressure had decreased by 14.1 mmHg and the diastolic pressure by 11.9 mmHg, compared with the starting value. At the end of the experiment both the systolic and the diastolic pressures stabilized towards the starting value but still remained slightly below it. Miller and Moor (1947) measured the blood pressure in patients suffering from neurosyphilis. After six hours in a room in which the starting temperature of +48.3°C was gradually decreased to +43°C—+44°C, there was no essential

change in the systolic blood pressure, whereas the diastolic pressure decreased by 25—50 mmHg. Blockley and Taylor (1948) observed an increase in the systolic blood pressure, but the direction of change in the diastolic blood pressure varied greatly in healthy young subjects kept in a recumbent position for 15—20 min. at an ambient temperature of +37.5°C—+121°C. Glaser (1950) compared the reactions of his subjects to a stay of 72 hours in temperatures of +4°C and of +40.5°C. In the hot room the systolic pressure was c. 10 mmHg below the corresponding values in the cold room, and the diastolic blood pressure 10—15 mmHg below. Wyndham (1951) reported that when a subject became accustomed to the hot environment the increase in the blood pressure was not as marked as during the first stage.

Changes in Venous Blood Pressure. — Daily and Harrison (1948) reported a maximal increase of 40 mmH_2O in the venous blood pressure of anaesthetized dogs in a hot environment. Scott *et al.* (1940) observed an increase from 10.9 cm. to 11.2 cm. H_2O in the venous blood pressure measured at the cubital vein, when healthy subjects remained recumbent at a temperature of +32°C. Henry and Gauer (1950) observed in human subjects that when the skin temperature in the foot had risen to temperatures between +36°C and +39°C, the venous blood pressure measured at the ankle did not fall below 70 mmH_2O, although the subject was walking. These investigators considered that the increase in the venous blood pressure was due to dilatation of the cutaneous vessels. Threefoot (1952) measured the venous blood pressure in subjects kept for 30 minutes in an ambient temperature of +40°C—+45°C and a relative humidity of 100%. The mean increase in the venous blood pressure was 40.9 mmH_2O in the cubital vein, 47.7 mmH_2O in the dorsal venous arch of the hand, and 35.7 mmHg at the dorsal venous arch of the foot.

Burch (1946 and 1953) observed that patients with cardiac failure are more liable to attacks of cardiac asthma in a hot and humid environment. In patients with cardiac failure Berenson and Burch (1952) observed a more marked dyspnea than in healthy subjects when exposed to a room temperature of +40°C and a relative humidity of 85% for a time varying between 40 and 114 min. The systolic blood pressure remained unchanged or decreased slightly, the diastolic pressure decreased, the superficial veins expanded and the cardiac murmurs increased. It is believed that an environ-

ment with a temperature of $+24°C$ and a humidity below 60% is healthiest for patients with cardiac disease.

Grollman (1930) investigated the effect on the circulation of pyrexia induced by injections of typhoid vaccine. The cardiac output, determined according to the acetylene method, increased soon after the injection, before any subjective symptoms had appeared in the patient. When the fever appeared, a decrease in the cardiac output could already be observed but the latter did not fall below basal values. Both the systolic and the diastolic blood pressures increased slightly. It has been demonstrated that pyrogenic agents tend to induce a fall in blood pressure in hypertensive patients. Chasis *et al.* (1942) reported a fall in the blood pressure of patients who had been treated with amidopyrine to eliminate the pyrexia symptoms. Thus, Page (1951) reported that induced pyrexia has been used as a treatment for malignant hypertension.

PREVIOUS INVESTIGATIONS INTO THE EFFECT OF THE SAUNA UPON CIRCULATION

Fey (1942) reported an increase in the pulse rate in the sauna, with a maximum rate of 164/min; v. Knorre (1943) reported a maximum rate of 120/min; Räsänen (1951) observed a mean pulse rate of 90/min. inmediately after the sauna while in the investigations of Eggers and Goll (1952) a maximum pulse rate of 138/min. was observed in the sauna. Klingler-Mandig (1952) found a mean pulse rate of 95/min. in 36 subjects who had been sitting in the sauna for 15—20 minutes. He did not observe any difference in the increase of the pulse rate between healthy subjects and patients with cardiovascular disease. The investigator does not mention the number of patients observed, nor the exact diagnosis of the disease (»Es handelte sich hier um Hypertoniker, Patienten mit Myokardschaden, Arterio- und Koronarsklerotiker oder um vagale Bradykardien»). According to Schröder and Eckhardt (1952) the pulse rate increased in the sauna from a value of 80/min. at rest to values of 150—160/min. Prokop (1953) reported a mean pulse rate of 120/min. after a 10 min. stay in the sauna. Luder (1954) observed a mean rise of 23.25% in the pulse rate. Ott (1948) found the mean pulse rate in 48 experiments in the sauna to be 124/min. and he observed different reactions of the pulse rate to the sauna. All subjects

tested showed an initial increase of 4—40 beats per min. during the first two minutes in the sauna, after which the pulse rate of some subjects advanced quickly to the maximum values of c. 130/min., whereas in others it reached maximum values only after 20 minutes in the sauna.

Polozow (1893) was probably the first investigator to mention the effect of the sauna upon the blood pressure. He measured the blood pressure in young healthy subjects while in the sauna, and observed a mean decrease in the systolic blood pressure of 10.4 mmHg. According to Lundgren (1933) these was an abrupt increase of 5—20 mmHg in the systolic blood pressure every time water was thrown on the hot stones. After the effect of the steam had passed, there was a slight decrease in the pressure and half an hour to an hour later the blood pressure had decreased to the starting values or slightly below these. The mean increase of the systolic pressure in the sauna was between 10 and 20 mmHg. Ott (1948) carried out blood pressure experiments on a group of 34 subjects. In subjects who remained recumbent while in the sauna the increase in the systolic blood pressure was as high as 15 mmHg. 9 out of 11 subjects who sat showed a mean increase of 13.5 mmHg in the systolic blood pressure; in one case no change was observed and in another there was a decrease of 5 mmHg. The diastolic blood pressure showed a mean increase of 8.5 mmHg during the first 2 minutes in 8 subjects, in two cases decreases of 5 and 10 mmHg were observed and in one case no change was observed. When water was thrown on the hot stones, small sudden rises were observed in the systolic blood pressure. According to Ott, subjects who show a slight increase or no change at all in the systolic blood pressure while in the sauna, are able to endure the hot environment well. He regarded such reactions as »normal», whereas a decrease in the systolic pressure indicated a pathological condition (». . . ausschliessliches Ansteigen des systolischen Druckes findet sich praktisch nur bei Personen mit guter Saunatoleranz, es kann als »normal» bezeichnet werden. — Ausschliessliches Abfall des systolischen Druckes tritt vor allem bei pathologischen Zuständen (Kollaps, latente Herzinsuffizienz) ein und kann nicht als normales Blutdruck verhalten während des Saunabades gewertet werden»). According to Ott again, the increase in the systolic blood pressure arose from a sympathicotonic condition caused by the

sauna. However, he considered the change in the blood pressure to be too small to exert any noteworthy strain upon the heart (»Wir haben während des Saunabades mit einer nennenswerten Mehrbelastung des Herzens durch Blutdrucksteigerung zu rechnen; jedoch sind die Steigerungen im allgemein nicht derart stark, dass dadurch der kritische Bereich des schlechten Wirkunggrades der Herzarbeit erreicht wird»).

Räsänen (1951) measured immediately after the sauna the blood pressure of a control group and of subjects with a history of myocardial infarction. In the control group there was a decrease in the systolic blood pressure in 55% of the tested subjects and an increase in 35%. The systolic pressure decreased in 75% and increased in 12.5% of the subjects with a history of myocardial infarction. In both groups the changes in the diastolic pressure followed fairly closely those in the systolic pressure.

Most investigators report a slight increase in the systolic blood pressure and a slight decrease in the diastolic pressure of healthy subjects while in the sauna. Hence the sauna can be expected to produce an increase in the pulse pressure (Fey 1942, Bardenheuer 1943, v. Knorre 1943, Karsten 1948, Devrient 1950, Schröder 1952 and Luder 1954). Bartels (1944) observed a decrease in the pulse pressure in the sauna. Klingler-Mandig (1952) observed a mean decrease of 12 mmHg in the systolic blood pressure of healthy subjects, and a mean fall of 19 mmHg in the systolic pressure of patients with cardiovascular disease. He made 65 determinations, of which 28 were on patients with cardiovascular disease, the same subjects being exposed to the sauna several times. The number of patients and the exact diagnosis were not given. The diastolic pressure did not change from the starting values. The subjects remained sitting during the experiments which lasted c. 20 minutes. Prokop (1953) reported a slight fall in the systolic pressure in the sauna and a brief rise after the sauna; the changes, however, were not statistically significant. Covalt (1954) found no essential change in the blood pressure in the sauna.

The author has not been able to find any literature on the effect of the sauna upon the circulation time. Gernandt *et al.* (1944) reported a fourfold increase in the cardiac output in the sauna when determined according to the method of Liljestrand and Zander. Ott, however, suggested an increase of c. 200—300%.

Klingler-Mandig (1952) observed an increase of 192% in the venous blood pressure immediately after the sauna. The experimental subjects were healthy and the increase was measured from the starting value of the venous pressure before the sauna.

Eggers and Goll (1952) demonstrated an increase of 200—300 cm^3 in the vital capacity in the sauna. Klingler-Mandig (1952) made 22 determinations of the vital capacity of healthy subjects in the sauna. 50% showed a mean decrease of 12% from the starting value, 25% showed no change and the remaining 25% a slight increase. Schröder and Eckhardt (1952) observed an initial increase of c. 17% in the vital capacity in the sauna, but 20 minutes later it started to fall. This fall was either uniform and of a mean magnitude of 410 cm^3, or periodical and uneven with a mean magnitude of 800 cm^3.

No marked electrocardiographical changes have been observed in the sauna. Lundgren (1938) observed a lowering of the T wave after the sauna in 35% of the tested subjects, but the form of the wave was not changed. The recording was done by means of limb leads. Ott (1947, 1948) carried out an electrocardiographic investigation in the sauna upon a series of 16 experimental subjects. He observed an increase of the P_2 and P_3 waves, a shortening of the PQ and the QRS intervals, a decrease of the R wave and the ST segment and also a decrease of the T_1 and T_2 waves. Ott believed that the electrocardiographic changes in the limb leads were due more to the sympathicotonia caused by the sauna than to myocardial anoxia. He based his views on experiments in which he could eliminate the electrocardiographic changes by giving an intravenous injection of dihydroergotamin to the subjects before the sauna. Eggers and Goll (1952) in their material consisting of 100 healthy subjects found c. 42% to be without electrocardiographic changes in the sauna. 58% of the subjects showed the following changes in the sauna: a decrease of the T_1 wave, a decrease of the T_1 and the T_2 waves, a decrease of the T_2 wave, a decrease of T_1—T_3, an increase of P_2, an decrease of ST_2 and supraventricular extrasystoles. All changes were reversible.

Räsänen (1951) recorded the electrocardiographic changes immediately after the sauna in a control group and in subjects suffering from myocardial infarction. The small changes which occurred were similar to those observed by Ott. Räsänen believed

that the slight electrocardiographic changes were primarily due to an increase in the pulse rate in the sauna. No additional changes in the electrocardiogram could be observed in the patients suffering from myocardial infarction. Klingler-Mandig (1952) could not observe any electrocardiographic changes in healthy subjects while in the sauna, nor could he find any additional changes in patients with cardiovascular disease.

THE PROBLEM

The number of investigations into the effect of the sauna upon the circulation is surprisingly small, and the results are partly controversial. Since the sauna is so widely used in Finland, there is also a practical importance in subjecting this problem to a systematic study.

The purpose of the present investigation was to answer the following questions:

1. Does the Finnish sauna give rise to changes — and if so, of what kind — in the cardiac output, the stroke volume, the circulation time, the intrathoracic blood volume, the pulse rate, the arterial blood pressure, the peripheral resistance and the venous blood pressure of healthy subjects?

2. If these changes take place, are they similar in hypertensive patients?

MATERIAL AND METHODS

CARDIAC OUTPUT

In this investigation an attempt has been made to measure as many hemodynamic factors as possible in each subject. An experimental method had to be found which made it possible to determine simultaneously several factors influencing the circulation.

The methods most widely used in determining the cardiac output are:

— Fick's Principle,

— to inject a certain amount of dye into the blood stream and to determine the concentration of the dye in the arterial blood at certain intervals,

— to inject radioactive iodine bound to albumin or

— to inject marked erythrocytes into the blood stream and to determine their concentration at certain intervals,

— to determine the cardiac output by the inspiration of foreign gases (e.g. acetylene).

The general formula for Fick's Principle is:

$$\text{cardiac output (cc)} = \frac{O_2 \text{ consumption (cc)}}{\text{arterial-venous } O_2 \text{ difference (cc\%)}} \times 100$$

The application of this method to sauna experiments would have been difficult. The necessity for cardiac catherization would have made it practically impossible to obtain volunteers; moreover, it was not possible to use X-ray equipment in the sauna. Hence, Fick's method could not be used.

Technically it would have been possible to determine the cardiac output by the acetylene method, but this method permits only one hemodynamic factor, the cardiac output, to be determined.

If the cardiac output is measured by the dye dilution method, other hemodynamic factors can be determined at the same time.

The equipment needed is simple and can, thus, be used under sauna conditions. Furthermore, it is easy to get volunteers for this painless method. In view of these advantages, the author decided to use the dye dilution method in his experiments.

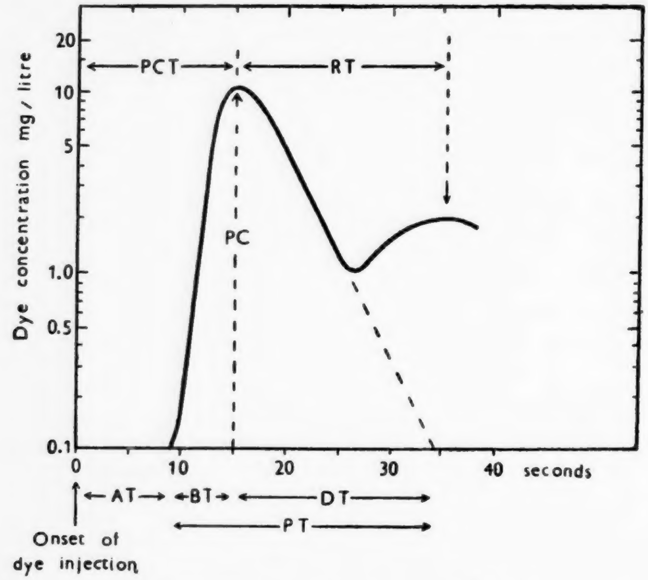

Fig. 1. — Dye-dilution curve. Time (in seconds) on the abscisse and concentration on the ordinate.

AT = Appearance Time, BT = Build Up Time, DT = Disappearance Time, PT = Passage Time, PCT = Peak Concentration Time, RT = Recirculation Time, PC = Peak Concentration.

The first man to use the principle of the dilution of an injected dye for determining cardiac output was Stewart (1897). He used a series of glass tubes in which he could calculate the volume flow (f) from the formula $f = \dfrac{I}{c_m \cdot t}$ where I = the amount of dye injected, and c_m = the mean dye concentration in the samples during the time t. This method could be used only when no recirculation occurred, so that its application to human subjects remained only a theoretical possibility. Hamilton *et al.* (1932) reopened the discussion of this problem. They showed that if the time and the concentration of the dye are plotted on a semilogarithmic chart, with the concentration indicated on the ordinate, the terminal part of

the resulting curve is a straight line. By extendiug, usually by eye, this straight line near to zero concentration, the effect of recirculation can be approximately eliminated (Fig. 1).

Many investigators (e.g. McMichael 1952, Doyle *et al.* 1953 and Dow 1955, 1956) have tested the accuracy of values for the cardiac output and the central blood volumes determined by the dye dilution method. A theoretical analysis of the method has also been made. The method has been proved to be fit for use. Comparisons between the cardiac output values obtained by the two methods have shown that the values obtained by Hamilton's method are practically comparable with those obtained by Fick's method (Hamilton *et al.* 1948, Werkö *et al.* 1949, Friedlich *et al.* 1950 and Eliasch 1952). Other investigations have been made in which the cardiac output has been determined simultaneously by the dye dilution method using Evans' Blue, and by the use of marked erythrocytes (Lawson *et al.* 1952). Freinkell *et al.* (1953) determined the cardiac output using albumin bound labelled iodine. The differences in the results obtained by these methods have been small and, practically, the results can be considered as identical.

Investigations have also been made into the influence of the site of the injection upon the dye concentration curve. The different time components in the dye concentration curve, e.g. the first appearance of the dye, the time for maximum concentration, the appearance of the recirculation etc., occur later when the dye is injected into a peripheral vein than when it is injected directly into the right auricle. The central blood volumes also increase when a peripheral injection site is used, but no significant changes have been observed in the cardiac output as measured by either method (Hetzel *et al.* 1954), although Lawson *et al.* (1954) indicated that the values for the cardiac output tend to be smaller when the dye injection is made into a peripheral vein than when made directly into the right auricle.

In this investigation all injections were made into a peripheral vein. The author justifies this by the fact that he compared the results taken in succession from the same subject at only small time intervals, and also by the results obtained by Hetzel *et al.* as mentioned above.

MEAN CIRCULATION TIME

Stewart (1897) was the first investigator who tried to determine the mean circulation time. Hamilton *et al.* (1932) developed a method suitable for the circulatory system. There has been considerable controversy as to the method of determining the mean circulation time from the dye dilution curve.

In a symmetrical curve the average transit time, the time coordinate of the peak and the time coordinate which halves the area all coincide. But as the curve becomes asymmetrical and particularly as the terminal portion occupies more time than the initial part, the three measures diverge more and more from each other. It has been shown that the replacement of the median by the mean made a difference of about 30% (Dow 1956). Meier and Zierler (1954) studied the error possibilities and worked out a formula for calculating the mean circulation time.

CENTRAL BLOOD VOLUMES

Once we know the mean circulation time and the volume of blood which flows in a given period of time along a given vessel, we can calculate the volume of that column of blood which lies between the point of injection and the point where the samples are collected (Stewart 1921).

If the dye is injected into a peripheral vein, the blood volume is called »the from needle to needle volume» or the intrathoracic blood volume. (Hamilton *et al.* 1932 and McMichael 1952). If the dye on the other hand is injected directly into the right auricle by means of a catheter, the blood volume is called the cardio-pulmonal blood volume (Ebert *et al.* 1949 and Lagerlöf *et al.* 1949).

Newman *et al.* (1951) determined the central blood volume from the direction of the decreasing part of the dye concentration curve. This part of the curve is a straight line, and hence the inclination of the curve can be determined by two known points on this line.

PERIPHERAL RESISTANCE AND MEAN ARTERIAL PRESSURE

Since the arterial blood flow in the peripheral vessels is fairly constant and uniform and there is relatively little turbulent flow, the peripheral resistance can be calculated by means of Poiseuille's

law (Reindell 1949). According to this principle the velocity of flow is directly proportional to the pressure difference and inversely proportional to the resistance.

Hence, $F = \frac{P - P_1}{R}$ and $R = \frac{P - P_1}{F}$

This formula, however, cannot be applied directly to hemodynamics. The resistance varies in each part of the periphery because of the higher resistance of the arterioles than of the capillaries, the elasticity and the irregular branching of the vessels. Hence every organ and every tissue has its own partial resistance (r_1, r_2 etc.). The total peripheral resistance is the sum of these parts and can be expressed theoretically by the following formula:

$$\frac{1}{TPR} = \frac{1}{r_1} + \frac{1}{r_2} + \frac{1}{r_3} + \ldots \frac{1}{r_n}$$ (Wiggers 1952).

The mean arterial blood pressure may be determined from the pulse curve by means of planimetry, but technical difficulties made it impossible to use direct determinations in this investigation and the author had to resort to the values obtained for the blood pressure. This method has frequently been used in other investigations, and due to the comparative character of this investigation absolute values are not necessary.

TEMPERATURES

It is not easy to find any special point in the organism which accurately expresses the general temperature of the body. Mead and Bonmarito (1949) tried to determine how accurately the rectal temperature reflects the general body temperature. They observed that when a rapid change from a warm environment to a cold environment occurs, the rectal temperature does not give a true picture of the general temperature change. For the purposes of this comparative investigation, however, the rectal temperature is an adequate indicator of the general body temperature changes that occur in the sauna.

Stoll and Hardy (1950) criticized the use of skin thermometers. They observed that the values vary according to the force by which the thermometer is pressed against the skin. In this investigation no mechanical apparatus was used to press the thermometer

against the skin, but an attempt was made to eliminate changes in the pressing force by using subjectively identical pressure every time.

METHODS USED

The sauna temperature was measured by an ordinary thermometer with an accuracy of 0.1°C placed at the height of the head of each subject. A wet bulb thermometer was used to determine the humidity of the air in the sauna. The accuracy of this thermometer was also 0.1°C. The rectal temperatures and the skin temperatures were determined by a Smith TE—3 thermometer with an accuracy of 0.1°C. All temperatures are reported in the centigrade scale.

The pulse rate was taken at the radial artery by palpation for one minute. The venous pressure was determined by puncturing the cubital vein kept at the height of the right atrium of the heart. The needle was held in the direction of the blood flow. The height of the column of blood was recorded in mmH_2O.

The systolic and the diastolic blood pressures were determined at the brachial artery by means of a manometer. The auscultatory method was used, the diastolic pressure being read at the moment when the Korotkoff sounds ceased.

The mean arterial blood pressure was determined by the following formula: $P_m = P_d + 0.42 \times P$ (Wezler and Böger 1939). P_d = the diastolic pressure, P = the pulse pressure.

The cardiac output was calculated from the formula, $f = \frac{I}{c_m t} \times \frac{100}{100 - H}$, where I = amount of dye injected, c_m = the mean arterial concentration of the dye during the time t, and $\frac{100}{100-H}$ = the hematocrit correction. The dye used was a 0.5% solution of Evans' blue in physiological saline. 5 ml. of the dye solution were measured into an accurately calibrated syringe. The dye which was left in the syringe after the injection was washed out with 10 ml saline solution. By a photometric determination of the concentration of the resulting solution it was possible to calculate the exact amount of dye injected.

The dye concentration in the plasma was determined either by a Coleman Spectrophotometer Model 14 or by a Beckman Spectrophotometer with micro cuvettes. The wave-length used was 625 mμ.

The results were plotted on a semilogarithmic chart with the concentration on the ordinate. Those cases in which it was impossible to draw a straight line through the concentration values on the falling part of the curve were discarded.

The hematocrit values were determined by using heparinized hematocrit tubes. The centrifugation time was 30 minutes with a speed of 4000 rev./min. The radius of the centrifuge was 10 cm.

The cardiac index was found by dividing the cardiac output by the body surface determined by Du Bois' formula (1916).

The stroke volume was determined by dividing the cardiac output by the pulse rate.

The mean circulation time was calculated according to the following formula:

$$T_m = \frac{\int_0^\infty t\, c_t\, dt}{\int_0^\infty c_t\, dt} \quad \text{(Meier and Zierler 1954)}$$

The following formula is used in practice:

$$T_m = \frac{\sum_0^w t \cdot c_t}{\sum_0^w c_t}; \quad c_t = \text{the concentration of the dye at the time } t.$$

The mean circulation time was taken in seconds between the superficial cubital vein in one arm and the brachial artery in the other arm.

The from needle to needle blood volume (Q) was calculated from the formula:

$$Q = \frac{\text{cardiac output}}{60} \times T_m \quad \text{(Hamilton 1932).}$$

The central blood volume (V) was calculated from the formula:

$$V = \frac{\text{cardiac output}}{S} \quad \text{(Newman 1951)}$$

S = the inclination of the falling part of the concentration curve. The central blood volume was in both cases determined in relation to the body surface.

The peripheral resistance was calculated from the formula:

$$R = \frac{\text{pressure fall in mm Hg} \times 9.81 \times 13.6}{\text{flow ml/sec.}} \text{ dyn. sec. cm}^{-5}$$

The peripheral vascular resistance in the systemic circuit was thus calculated as

$$R = \frac{\text{brachial arterial mean pressure}}{\text{cardiac output ml/sec.}} \times 1332 \text{ (Eliasch 1952).}$$

MATERIAL

The group of healthy subjects consisted of males in good physical condition, most of them active athletes, used to the sauna and to hot baths. The history of none of this group revealed anything suggesting cardiovascular disease, and none of them showed signs of disease in a clinical checkup before the experiment. Their age ranged between 22 and 55 years.

The group of hypertensive subjects came from the I and III Medical Clinic of the University Hospital and from the Aurora Hospital in Helsinki. Their age ranged between 39 and 51 years. All subjects suffered from essential hypertension. No patient with signs of cardiac failure was included. The hypertensive patients in whom only the blood pressure was determined in the sauna had previously been under medical treatment in the above mentioned hospitals or with private practitioners. In the pre-experimental clinical checkup no signs of cardiac failure could be observed in this group.

The total material consisted of 25 healthy subjects and 8 hypertensive patients in whom the cardiac output, circulation time, pulse rate, stroke volume, arterial blood pressure, peripheral resistance and the venous blood pressure were determined before the sauna and in the sauna. In addition, the blood pressure of 20 other hypertensive patients of ages ranging between 22 and 55 years was taken before the sauna, in the sauna and after the sauna.

PERFORMANCE OF THE INVESTIGATION

All measurements before the sauna were taken under as basal conditions as possible. The subject was weighed and then rested for 45 min. in a recumbent position in the dressing room, the temperature of which ranged between 18 and 20°C. During this time the brachial artery was punctured with a special artery needle, which remained in place throughout the experiment. After 45 minu-

tes the pulse rate, the rectal temperature, the venous blood pressure, the systolic and the diastolic blood pressures were taken. The dye was then immediately injected into the superficial cubital vein with the arm of the subject raised 70° from the horizontal. The injection was performed as quickly as possible (c. 1 sec.). From the moment the syringe was empty the time was taken with a stop-watch registering 2-second intervals. The mandrin was removed from the artery needle and the blood allowed to flow freely. The test tubes were kept in a holder and every other second a new tube was placed under the flow. The collection time for all samples ranged between 52 and 56 sec. The samples were left standing for a few minutes and were then centrifuged for 20 min.

One hour after the dye injection the subject entered the sauna, and again took up a recumbent position. The bathing time and the temperature of the sauna varied according to the endurance of the subject, who was allowed to get used to the hot environment before water was thrown on the hot stones at his own request. The skin, rectal temperatures and sauna temperatures were read simultaneously three times during the stay in the sauna, the last reading being taken when the subject reported that he had bathed enough. Immediately after this the dye was injected into the vein at the same place as before the sauna. The amount of dye injected and the time taken by injection were also the same as previously. The collection time in the sauna was 36 sec. The pulse rate, the arterial blood pressure and the venous blood pressure were then determined, after which the subject returned to the dressing room where he was dried and weighed. The hypertensive subjects rested in a recumbent position for 20 min. during which their blood pressure was measured again.

The blood pressure of 20 hypertensive patients was observed in the sauna at 5 min. intervals. These subjects also lay in a recumbent position throughout the experiment. Water was thrown on the hot stones at the subject's request. The sauna temperatures were taken at the same time as the first and the last blood pressure determinations were made. The subjective condition of each subject was closely observed throughout the experiment.

After the sauna these hypertensive patients rested in the dressing room for one hour, during which their blood pressure was measured at 20 min., 30 min. and 60 min. intervals after the sauna.

STATISTICAL METHODS

A statistical analysis of the material has been performed according to the methods suggested by Fisher (1950).

Effect of the Qualities of the Sauna on the Observations. — Before proceeding to a detailed analysis it had to be decided whether the numerical observations were dependent on such qualities of the sauna as the temperature, the wet bulb temperature and the duration of the bath. If so, the effect of the sauna qualities would have to be eliminated from the numerical observations and the reduced data subjected to closer statistical analysis.

In this investigation *multiple regression analysis* was applied. In this type of analysis it is assumed that the statistical variable under investigation, x_0, is, on an average, a linear function of one or more explanatory variates x_1, x_2, x_3 *etc.*, thus:

$$x_0 \simeq a + b_1x_1 + b_2x_2 + b_3x_3 + \dots \tag{1}$$

The variates actually used in the right-hand member of this equation are:

x_1 = temperature, degrees centigrade,
x_2 = duration of the bath, minutes, and
x_3 = wet bulb temperature, degrees centigrade.

The relative cardiac index, *i.e.* the value of the cardiac index in the sauna expressed as a percentage of that before the bath, was chosen as the statistical variate, x_0, to be investigated. This variate alone is considered sufficient to provide information on the effect of the sauna qualities on the observations. If no effect is found on the relative cardiac index, the qualities of the sauna are unlikely to have any noteworthy effect on other measures of cardiac function. Of course, this reasoning is valid only for the range of temperature, of the duration of the bath and of the wet bulb temperature observed in these experiments.

As usual, the constants in equation (1) have been determined by applying the *method of least squares*. The results of the calculations are as follows:

Constant	Calculated value	Standard error
a	43.0	15.6
b_1	–0.22	1.76
b_2	0.95	2.84
b_3	2.98	3.99

The magnitude of the standard errors indicates that none of the b-coefficients is statistically different from zero. Thus, *linear dependency cannot be statistically established.* This is confirmed by the following *analysis of variance:*

Source of variation	Sum of squares	Degrees of freedom	Variance	Variance ratio
Regression	3619	3	1,206	[4.62]
Residual	111375	20	5,569	.
Total	114994	23	..	.

The proportion of the total sum of squares »explained» by the regression equation is negligible, and thus no significant regression exists.

This analysis indicates that the qualities of the sauna need not be taken into account in further study of the material. Although the results may have some material dependency on these qualities, such a dependency is rather unlikely to have a statistically important effect. In other words, the numerical observations are probably affected more by other relevant factors than by the sauna qualities; indeed, the role of other factors seems to be essential.

Statistical Analysis of Changes Effected by the Sauna. — For a general description of the observations made both before the sauna and during it, the *mean*

(2) $\bar{x} = \Sigma x/n$

has been calculated. In the formula, x stands for the observed values of each variate, n being the number of observations. Computations of the dispersion of the variates are based on the *sum of squares*

(3) $Q = \Sigma x^2 - (\Sigma x)^2/n.$

The *variance* or the squared *standard deviation* is estimated according to the formula

(4) $s^2 = Q/(n-1),$

and the *standard error of the mean* according to the formula

(5) $s_{\bar{x}} = s/\sqrt{n}.$

The general trend of the changes is described by the mean of the individual changes. In calculating the standard error of this mean, *correlation* between observations taken before and during

the sauna has been observed. In practice, the procedure is as follows. Denoting x_1 for observations made before the sauna-bath and x_0 for observation made during the bath, the sum of squares of changes equals

(6) $$Q_{01} = Q_0 + Q_1 - 2S_{01},$$

where the *product sum*

(7) $$S_{01} = \Sigma x_0 x_1 - (\Sigma x_0 \cdot \Sigma x_1)/n$$

depends on the correlation mentioned above. After computing the sum of squares in (6), further calculations concerning the dispersion of the changes can be performed according to common formulas. Thus, the standard error of the mean change, $s_{\bar{x}_0} - _{\bar{x}_1}$, can be estimated.

On the basis of these calculations the *t*-test can be made as follows:

(8) $$t = (\bar{x}_0 - \bar{x}_1)/s_{\bar{x}_0 - \bar{x}_1}.$$

By consulting appropriate statistical tables (*e.g.* Fisher & Yates 1953) it can then be determined how large is the probability of obtaining, by chance only, a difference equal to or larger than that recorded between the observations made before the sauna and during it. In a similar way, *mutatis mutandis*, the differences of the changes in the sauna between healthy subjects and hypertensive patients have also been tested.

In reporting the results of the tests, the *statistical significance* of the conclusions is indicated by asterisk symbols as follows:

- * if the (chance) probability mentioned above is not larger than 5 per cent (»almost significant»),
- ** if this probability is not larger than 1 per cent (»significant»), and
- *** if the probability equals at most 1 pro mille (»highly significant»).

In estimating »true» average changes effected by the sauna, a region of inaccuracy is to be attached to the means actually observed. The magnitude of such a region can be estimated by multiplying the standard error of any mean by the *t*-value corresponding to the number of degrees of freedom available and to the desired *level of confidence*. This latter expression refers to the probability that the region thus determined (usually called the *confidence interval*) would include also the »true» mean. For example, if 95 per cent

confidence is required in the present material, the critical t-value equals 2.07 for the group of healthy subjects (23 degrees of freedom), and 2.36 for the hypertensive group (7 degrees of freedom). The standard error of the mean multiplied by these t-values, plus/minus the mean, gives the confidence interval desired.

In examining the changes effected by the sauna, the numerical dependence of the observations made in the sauna on those made before has also been analyzed. The results of this *correlation analysis* are given in the form of an *efficiency percentage* E (Kihlberg 1955) which is calculated as follows:

(9) $$E = 100\ (1 - \sqrt{1 - r^2}).$$

In this formula,

(10) $$r^2 = S_{01}^2 / Q_0 Q_1$$

stands for the squared *correlation coefficient*. The efficiency percentage indicates how large a proportion of the range of variability of the observations made in the sauna can be »explained» as due to a corresponding variability before the sauna. In giving actual values of E, the asterisk symbols described above have been used. In this case, the symbols indicate the statistical significance of the correlation under investigation.

If the correlation of the observations made in the sauna (x_0) with those made before (x_1) is not negligible, it may be of value to know the numerical values of the constants a and b in the *regression equation*

(11) $$x_0 \simeq a + bx_1.$$

As usual, these values have been determined by applying the method of least squares. The constants reveal the location and slope of the *regression line* shown in the graphs. The regression line, on the other hand, shows schematically the expected values of observations in the sauna for given values observed before.

In the graphs, the horizontal axis shows observations made before the sauna, and the vertical axis observations made in the sauna. The diagonal line represents observations which are equal both before the sauna and in it. If there is an increase during the bath, the observation points will lie above the diagonal; if there is a decrease, below it. Regression lines have been calculated and shown separately for healthy subjects and hypertensive patients.

Further Interdependence Analyses. — In investigating the interrelations of two observed variates, *e.g.* the pulse rate (x_0) and the rectal temperature (x_1), it was assumed that formula (11), on an average, provides a suitable mathematical model, *i.e.* it was assumed that the relation under investigation was linear. By applying the method of least squares, the constants a and b of (11) were then determined together with the efficiency percentage E defined in formula (9). The statistical significance of the dependence studied is again indicated by asterisk symbols.

The graphical presentation of these interdependence analyses is, in essence, similar to that described above.

Summary Diagram. — In the summary diagram (Fig. 15) a common logarithmic scale has been used in order to render the observations comparable, in the sense that proportionally equal changes are always shown as equally large whatever the numerical values of the variates studied may be.

RESULTS

The time spent in the sauna by the healthy subjects varied according to their endurance between 14 and 35 min., the average time being 21 min. The temperature of the sauna at the moment the tests were taken varied between +57°C and +86°C while the wet bulb temperature was between +38°C and +51°C. The mean sauna temperature was +69.4°C on the dry bulb thermometer and +44.3°C on the wet bulb thermometer.

The skin temperature of healthy subjects in the sauna at the moment the tests were taken was between +41.6°C and +38.1°C, with an average of +39.7°C. As mentioned before, the values for the skin temperature are not absolute, but as they were all taken in a similar way, they should at least give a comparative picture of the temperature changes occurring at the skin of the test subjects while in the sauna. The rectal temperature at the moment the tests were taken varied between +37.7°C and +39.2°C, with an average of +38.2°C.

In the healthy subjects the rectal temperature has been correlated with some other results obtained in the sauna. There was no significant correlation between the rectal temperature and the stroke volume in the sauna ($E = 3.2\%$), between the rectal temperature and the cardiac index measured in the sauna ($E = 7.5\%$), or between the rectal temperature and the wet bulb temperature ($E = 0.5\%$).

On the other hand, a highly significant correlation was observed between the rectal temperature and the dry bulb temperature in the sauna (Fig. 2).

There was also a significant correlation between the rectal temperature and the time spent in the sauna (Fig. 3). The values for the hypertensive subjects who were tested by the same methods as the group of healthy subjects are also shown in the figure.

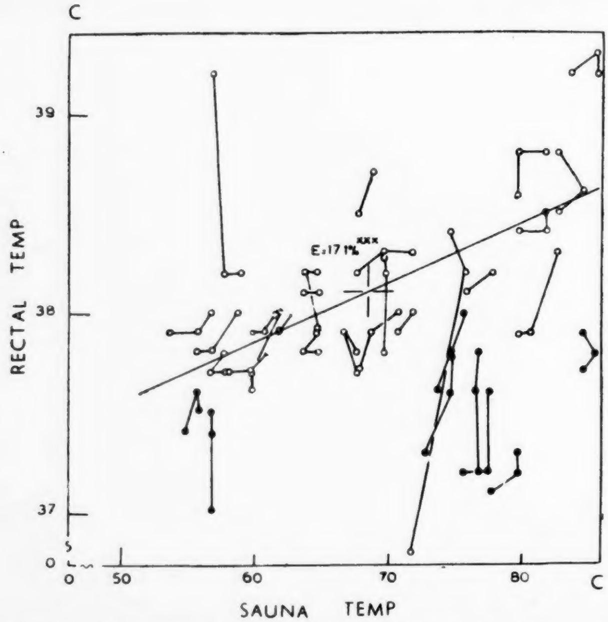

Fig. 2. — Graph showing the correlation of the rectal temperature and the sauna temperature, together with a regression line expressing the average relation for healthy subjects. Three determinations, the dots connected by straight lines, have been made for each subject. Regression line reveals evident correlation between the temperatures.

O = healthy subjects, ● = hypertensive patients, + = average for healthy subjects, — = regression line for healthy subjects, E = efficiency percentage for healthy subjects.

The hypertensive subjects stayed in the sauna from 10 to 23 min. the average time being 15.5 min. Hence these subjects bathed on an average 5.5 min. less than the healthy subjects. The sauna temperature for the hypertensive patients at the moment the tests were taken varied between +56°C and +85°C (average +73°C), while the wet bulb readings were between +38°C and +50°C (average +44.4°C). Thus, the dry bulb temperature was 3.6°C higher than in the tests with healthy subjects, while the wet bulb temperature stayed practically unchanged. The skin temperature of the hypertensive patients at the moment the tests were taken varied between +38.0°C and +41.3°C with an average of +39.5°C. According to these results the skin temperature was only 0.2°C lower in the hypertensive patients.

The rectal temperature in the hypertensive patients at the

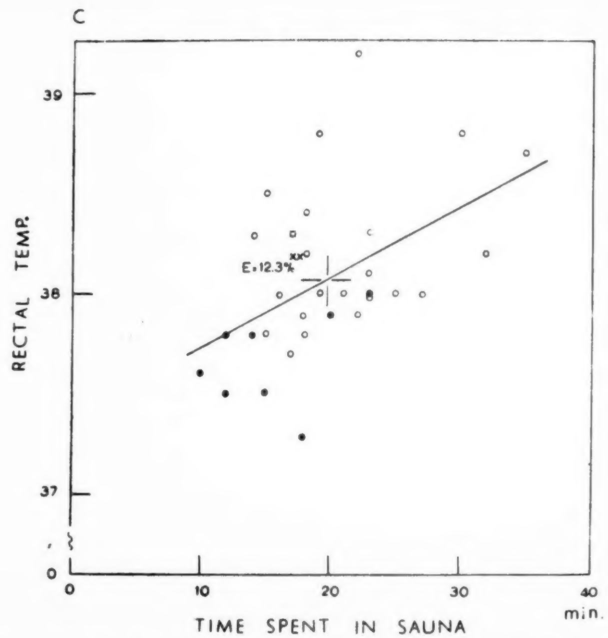

Fig. 3. — **Graph showing the correlation of the rectal temperature (final determination in the sauna) and the time spent in sauna, together with a regression line expressing the average relation for the whole material. Regression line reveals evident correlation between the variables studied.**

O = healthy subjects, ● = hypertensive subjects, + = average for the whole material, — = regression line for the whole material, E = efficiency percentage for the whole material.

moment the tests were taken varied between +37.3°C and +38.0°C, with an average of +37.7°C. This was 0.5°C lower than in healthy subjects.

The hypertensive patients of whom only the blood pressure was taken stayed in the sauna for 15 min. with the exception of one patient. The dry bulb temperature reached a value between +67°C and +92°C in 5 minutes (average +79°C) and at the end of the bath the dry bulb temperatures ranged from +69°C to +90°C (average +79°C). The values for the wet bulb temperature were between +31°C and +41°C (average +35°C) in 5 minutes and the same (average +36°C) at the end of the bath period.

BLOOD PRESSURE MEASUREMENTS

Systolic Blood Pressure. — Large individual variations were found in the systolic blood pressures (Fig. 4).

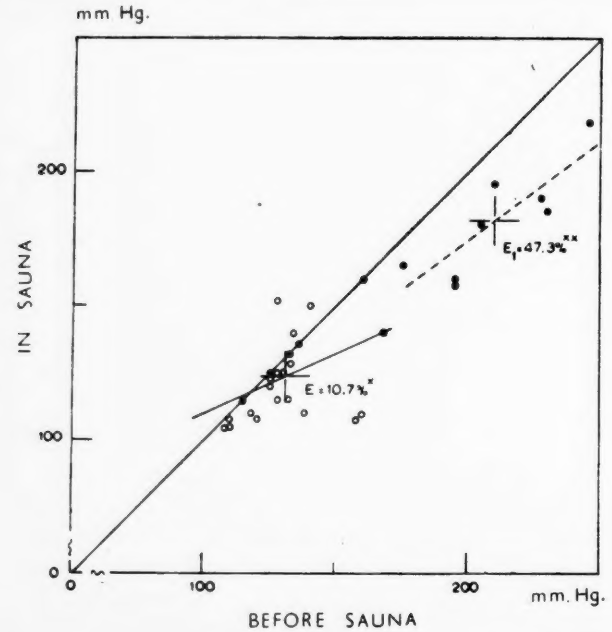

Fig. 4. — **Graph showing individual determinations of the systolic blood pressure before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant decrease in the systolic blood pressure; regression lines reveal positive correlation of the systolic pressure in the sauna on that before the sauna.**

O = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

Before the sauna the systolic pressure varied between 108 and 168 mmHg, the mean systolic pressure being 132 mmHg. In the sauna the individual results showed marked dispersion. The highest systolic pressure in the sauna was 160 mmHg and the lowest 105 mmHg, the mean value being 124 mmHg (Table 1).

In healthy subjects the predominant change was a fall in the systolic blood pressure in the sauna compared with the corresponding values before sauna. The greatest fall in pressure was 50 mmHg. Rises in the systolic pressure were generally insignificant with the exception of one case in which the systolic pressure was 24 mmHg higher in the sauna than before. The systolic pressure in the sauna was on an average 8 mmHg or 6% lower than before the sauna. This change was almost significant statistically, but it was small in

TABLE I

SYSTOLIC BLOOD PRESSURE (mmHg) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS [1]	Healthy	HS	Healthy	HS
Range	108—168	175—246	105—160	158—218	-50— +24	-45— -10
Mean	132	210	124	181	-8	-29
Standard error of the mean	3.3	8.2	3.2	7.2	3.4	4.2
Percentage change from values before sauna					-6	-14
Testing the significance of the changes: t					2.40*	6.85***
Testing the significance of the difference [2] in the change: t					3.83***	

[1] HS = hypertensive subjects
[2] difference = the mean difference of the change in systolic blood pressure between healthy subjects and hypertensive subjects.

size and would have no practical significance in individual cases. Comparisons between direct and indirect measurements of the blood pressure have been found to give markedly different values for the systolic pressure (Roberts *et al.* 1953 and van Bergen *et al.* 1954). The differences ranged from 10 to 20 mmHg, the direct method giving the higher values. On the other hand Hamilton *et al.* (1936) reported no significant differences between the values obtained by the direct and the indirect methods of blood pressure measuring. If we compare the values for the systolic blood pressure before and during the sauna it will be found that they show a statistically almost significant correlation (Fig. 4).

The mean value for the systolic blood pressure of the hypertensive patients was 210 mmHg. The pressure varied between 175 and 246 mmHg (Table I). In the sauna the mean value for the systolic pressure was 181 mmHg. In all cases there was a marked fall in the systolic blood pressure during the stay in the sauna (Fig. 4), the largest being 45 mmHg and the smallest 10 mmHg. The change was highly significant statistically. The correlation between the values for the systolic blood pressure before and during the sauna was also statistically significant (Fig. 4). The direction of change in the systolic blood pressure was, thus, much clearer in the hypertensive patients than in the group of healthy subjects. The difference be-

tween the mean change in the healthy subjects and the hypertensive patients was also highly significant statistically.

Diastolic Blood Pressure. — Table II shows the results of measurements of the diastolic blood pressure. It can be observed in Fig. 5 that the diastolic pressure taken with the subject at rest also shows a wide range of values.

TABLE II

DIASTOLIC BLOOD PRESSURE (mmHg) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE SUBJECTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	60—104	110—130	60—104	80—120	-20—+15	-30—0
Mean	80	119	74	106	-6	-14
Standard error of the mean	2.1	2.9	1.9	4.4	1.7	4.0
Percentage change from values before sauna					-8	-11
Testing the significance of the change: *t*					3.53**	3.47**
Testing the significance of the difference in the change: *t*					1.77	

The changes took different directions. The mean fall in the sauna was -6 mmHg compared with the starting value; this means a fall of 8%. This change was statistically significant. The fall, however, is very small, and seems to have no more practical significance than the change in the systolic blood pressure. Fig. 5 shows a highly significant correlation between the values for the diastolic blood pressure before and during the sauna.

Although the direction of change in the diastolic blood pressure in the sauna was clearer in the hypertensive subjects than in the healthy subjects, no statistically significant difference between the changes in these two groups could be shown (Table II). The diastolic pressure before the sauna varied between 110 and 130 mmHg, the mean value being 119 mmHg. In the sauna the mean value was 106 mmHg or 11% smaller than the starting value. This change was statistically significant. The maximum fall was 30 mmHg, and no rises in pressure were observed in any of the subjects tested. There was no correlation between the results obtained before the sauna and during the sauna (Fig. 5). The systolic and diastolic

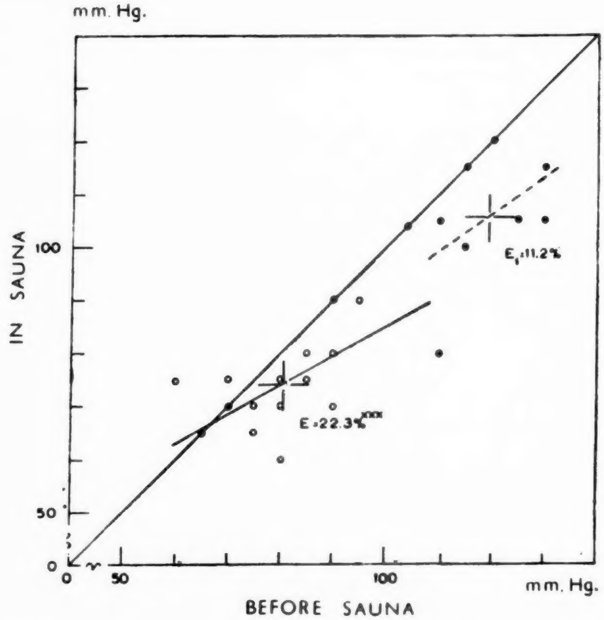

Fig. 5. — Graph showing individual determinations of the diastolic blood pressure before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant decrease in the diastolic blood pressure; regression lines reveal significant correlation of the diastolic pressure in the sauna on that before the sauna for healthy subjects.

O = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

blood pressures of the hypertensive patients continued to fall after the sauna (Table III).

A fairly clear falling tendency was observed in both the systolic and diastolic blood pressures, the highest fall being 80 mmHg for the systolic blood pressure and 55 mmHg for the diastolic blood pressure compared with the starting values. In the case of the systolic pressure 35 mmHg was the smallest fall observed. The change from the starting value was —27% for the systolic blood pressure and —25% for the diastolic pressure. The pulse pressure decreased in all cases on an average by 29%. Hence the decrease in the blood pressure which started in the sauna continued after the sauna. The decrease in both the systolic and the diastolic blood pressure after the sauna was of a similar magnitude to that which had occurred in the sauna.

TABLE

BLOOD PRESSURE VALUES (mmHg) FOR HYPERTENSIVE SUBJECTS[1]

	Systolic Pressure		
	Before Sauna	In Sauna	After Sauna
Range	175—246	158—218	110—175
Mean	205	176	151
Standard error	7.36	5.68	8.84
Change from values before sauna			
Range	.	–45——10	–80——35
Mean	.	–29	–54
Standard error	.	4.25	5.91
t	.	6.85***	9.14***
Change from values in sauna			
Range	.	.	–55——10
Mean	.	.	–25
Standard error	.	.	5.88
t	.	.	4.20**

[1] These values differ from the values in Table I because one patient's

As compared with the values obtained in the sauna the fall of the systolic blood pressure after the sauna was significant, and the change in the pulse pressure was almost significant, but the fall of the diastolic blood pressure after the sauna was not significant. If the results are compared with the starting values, the fall in the systolic blood pressure is highly significant, and the changes in the diastolic blood pressure and in the pulse pressure significant. It appears that the sauna has a marked reducing effect upon the blood pressure of hypertensive patients.

Mean Arterial Blood Pressure and Pulse Pressure. — Before the sauna the mean arterial blood pressure varied between 86 and 131 mmHg (Table IV).

The values for healthy subjects in the sauna were on the average 7% lower than the corresponding values before the sauna. The fall in pressure was highly significant statistically. The mean fall, however, was only 7 mmHg. A highly significant correlation between the values for the mean arterial blood pressure as measured before and during the sauna was obtained ($E = 25.8\%$***).

The systolic and the diastolic blood pressures of the hypertensive subjects decreased during the sauna in all cases; hence the

III

BEFORE, DURING AND 20 min. AFTER THE SAUNA

Diastolic Pressure			Pulse Pressure		
Before Sauna	In Sauna	After Sauna	Before Sauna	In Sauna	After Sauna
110—130	80—120	60—115	60—126	50—98	50—75
119	104	90	86	73	61
3.35	4.46	6.81	5.95	5.91	2.83
.	–30—0	–55——10	.	–30——5	–40——10
.	–14	–29	.	–15	–25
.	3.98	7.02	.	3.21	4.48
.	3.47**	4.17**	.	4.80**	5.51**
.	.	–55—+10	.	.	–25—+7
.	.	–14	.	.	–11
.	.	9.11	.	.	4.24
.	.	1.49	.	.	2.64*

blood pressure was not recorded after the sauna.

mean arterial blood pressure was also lower in the sauna than before the sauna (Table IV). The fall was more marked in the hypertensive subjects than in the healthy subjects, there being a significant difference between the healthy subjects and the hypertensive patients in the changes of the mean arterial blood pressure. The values for the mean arterial blood pressure varied between 140 and 173 mmHg in hypertensive patients. The mean value before the sauna was 158 mmHg and in the sauna 137 mmHg, giving a decrease

TABLE IV

MEAN ARTERIAL BLOOD PRESSURE (mmHg) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	86—131	140—173	79—119	114—161	–33— +4	–32— –4
Mean	102	158	95	137	–7	–21
Standard error of the mean	2.4	4.5	1.8	4.98	1.8	3.8
Percentage change from values before sauna					–7	–13
Testing the significance of the change: t					3.92***	5.45***
Testing the significance of the difference in the change: t					3.30**	

of 13%. The change was statistically highly significant. The maximum fall was 32 mmHg. The mean arterial blood pressure rose in none of the subjects. There was no correlation between the values obtained before and after the sauna ($E = 26.7\%$, $0.05 < P < 0.1$).

The pulse pressure measured in the sauna showed no great change compared with the starting values in healthy subjects (Table V).

TABLE V

PULSE PRESSURE (mmHg) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	30—75	60—126	18—85	50—98	–50—+34	–30—–5
Mean	51	91	49	76	–2	–15
Standard error of the mean	2.6	7.17	3.6	6.02	3.8	3.21
Percentage change from values before sauna					–4	–17
Testing the significance of the change: t					0.55	4.80**
Testing the significance of the difference in the change: t					2.67*	

The pulse pressure was on the average only 2 mmHg or 4% smaller in the sauna than before the sauna. The decrease is not statistically significant, nor was there any statistically significant correlation between the starting values and the sauna-values of the pulse pressure ($E = 3.7\%$).

On the other hand, all hypertensive patients tested showed a pronounced decrease in the pulse pressure values in the sauna (Table V). The decrease varied between 30 and 5 mmHg, with an average of 15 mmHg, the change being statistically significant. The percentage change was —17% in the hypertensive patients compared with —4% in the healthy subjects. There was an almost statistically significant difference between the mean fall in the pulse pressure of the healthy subjects and of the hypertensive patients. In the hypertensive patients there was a significant correlation between the values obtained before the sauna and in the sauna ($E = 56.4\%$**).

Peripheral Resistance. — In the healthy group the peripheral resistance decreased by 42% in the sauna (Table VI).

TABLE VI

PERIPHERAL RESISTANCE (DYN.SEC. CM^{-5}) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	1056—3419	1268—3994	468—1873	722—2724	–1733—+671	–1290—-522
Mean	1611	2184	931	1179	–680	–1005
Standard error of the mean	98	327	70	231	88	144
Percentage change from values before sauna					–42	–46
Testing the significance of the change: t					7.72***	6.98***
Testing the significance of the difference in the change: t					1.92	

Before the sauna the mean peripheral resistance was 1611 dyn. sec. cm^{-5}. Eliasch (1952) reports that the normal value for the peripheral resistance is between 700 and 1900 units, when the mean arterial blood pressure is measured according to the direct method.

The change in the peripheral resistance in the sauna was highly significant. It was also possible to show a statistically almost significant correlation between the values obtained for the peripheral resistance before and in the sauna (Fig. 6).

The peripheral resistance of the hypertensive patients before the sauna varied between 1268 and 3994 dyn. sec. cm^{-5} with a mean value of 2184 dyn. sec. cm^{-5} (Table VI). In the sauna the mean peripheral resistance was 1179 units, the greatest fall being 1290 units and the mean fall 1005 units. A highly significant correlation was also observed between the values for the peripheral resistance before and in the sauna (Fig. 6). There was no difference between the healthy and the hypertensive subjects in the changes of the peripheral resistance.

Systolic, Diastolic and Pulse Pressures (mmHg) in That Group of Hypertensive Subjects in Which the Blood Pressure Only Was Measured. — This group of hypertensive subjects was found to have systolic blood pressure values before the sauna ranging between 160 and 260 mmHg, with an average of 209 mmHg. The diastolic pressure ranged between 70 and 140 mmHg, with an average of 116 mmHg (Table VII).

Both the systolic and the diastolic pressure showed a tendency to fall after only 5 min. in the sauna (Fig. 7).

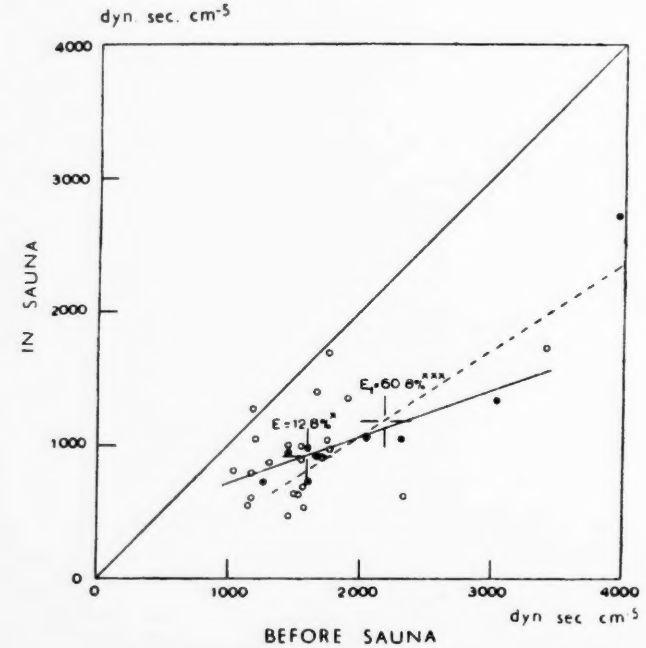

Fig. 6. — Graph showing individual determinations of the peripheral resistance before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant decrease in the peripheral resistance; regression lines reveal significant correlation of the peripheral resistance in the sauna on that before the sauna.

O = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

TABLE VII

THE BLOOD PRESSURE (mmHg) BEFORE, DURING AND AFTER THE SAUNA IN THE HYPERTENSIVE PATIENTS IN WHICH THE BLOOD PRESSURE ONLY WAS MEASURED

	Before Sauna	In Sauna			After Sauna		
		5 min	10 min	15 min	20 min	30 min	60 min
Systolic blood pressure							
Range	160—260	115—230	145—230	140—220	130—210	130—195	145—22[illegible]
Mean	209	186	180	181	170	166	174
Diastolic blood pressure							
Range	70—140	70—140	70—140	65—145	50—140	60—140	60—14[illegible]
Mean	116	110	104	101	100	101	97
Pulse pressure							
Range	70—125	45—120	55—110	50—115	40—140	35—115	45—12[illegible]
Mean	94	76	76	80	70	64	72

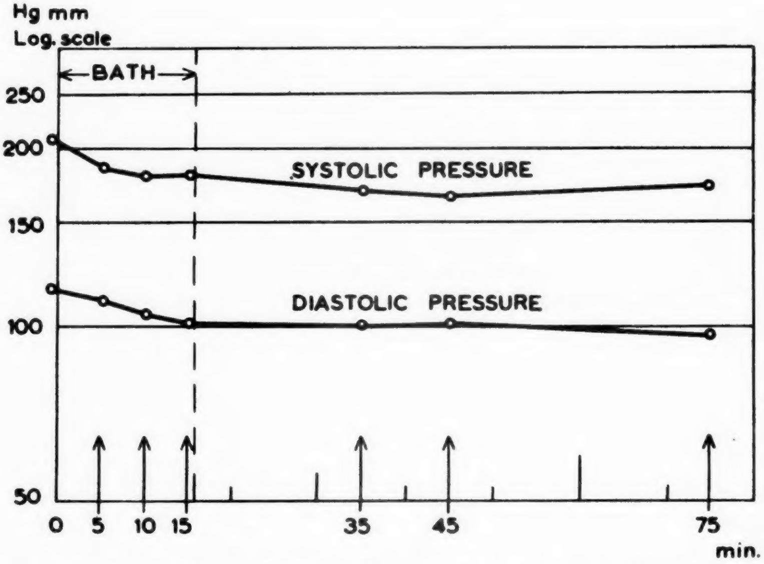

Fig. 7. — Graph showing the average systolic and diastolic blood pressure curves for hypertensive patients for whom the blood pressure only was determined.

In 3 cases out of 20 was there no noticeable change in the systolic pressure after 5 min. in the sauna. In two cases, however, the systolic blood pressure fell 30 and 35 mmHg in 10 minutes and in one case 30 mmHg in 15 minutes. Two cases showed a slight rise in the systolic pressure during the first 5 minutes in the sauna.

In one of these subjects the rise in the systolic blood pressure was 5 mmHg; subsequently the pressure decreased to a value below the starting value, and then returned to the starting value (205 mmHg) at the end of the bath. This subject's diastolic blood pressure on the other hand decreased 5 mmHg in 5 minutes and stayed below the starting value during the whole experiment.

The other subject was the only one in the whole group whose systolic blood pressure remained above the starting value during the entire bath. In the sauna his systolic pressure was between 5 and 15 mmHg higher than the starting value (160 mmHg). This diastolic pressure also rose after 5 min. in the sauna from 110 mmHg to 120 mmHg, but fell back to the starting value at the end of the bath. This subject had stenocardiac pain lasting for c. ½ min. at the beginning of the bath, but the pain did not recur. He

was the only subject to experience pain or subjective discomfort during the sauna.

In four cases the diastolic blood pressure remained unchanged after 5 minutes in the sauna; in two cases it increased. One of these two cases has been described above; in the other the pressure rose from 80 mmHg to 85 mmHg.

At the end of the sauna the systolic blood pressure had decreased 28 mmHg on the average, the greatest fall being 90 mmHg as compared with the starting value. The change was highly significant statistically (t = 5.40***). The diastolic blood pressure had decreased 15 mmHg on the average at the end of the sauna, the greatest fall being 40 mmHg. The change in the diastolic pressure was also highly significant (t = 4.82***). The pulse pressure decreased 14 mmHg on the average compared with the starting value. This change was significant (t = 3.19**).

In none of the cases tested one hour after sauna had the systolic blood pressure risen above the starting value; in two subjects it was the same as before the sauna. The mean decrease in the systolic pressure one hour after the sauna was 35 mmHg, the greatest fall being 70 mmHg. The decrease was highly significant statistically (t = 7.80***). One hour after the sauna the diastolic blood pressure had increased 5 mmHg above the starting value in one case, and the same value as at the start was obtained one hour after the sauna in two cases. The mean fall in the diastolic blood pressure measured one hour after the sauna was 19 mmHg. The change was statistically significant (t = 6.73**). The decrease in the pulse pressure measured one hour after the sauna was more marked than when measured just after the bath. The mean decrease was 22 mmHg compared with the starting value. This change was highly significant statistically (t = 5.88***).

Venous Blood Pressure. — The venous blood pressure rose in all healthy subjects (Table VIII). The change varied between +8 and +128 mmH_2O.

The rise in the venous blood pressure was highly significant statistically. Fig. 8 shows the changes in the venous blood pressure in individual cases. It was possible to show a significant correlation between the values for the venous pressure before and during the sauna.

In the hypertensive patients the venous blood pressure also

TABLE VIII

VENOUS BLOOD PRESSURE (mmH_2O) MEASURED IN THE CUBITAL VEIN IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	33—119	40—113	47—218	62—163	+8— +128	+1— +50
Mean	62	68	99	96	+37	+28
Standard error of the mean	4.8	7.6	7.9	12.1	6.5	6.2
Percentage change from values before sauna					+60	+41
Testing the significance of the change: t					5.76***	4.47**
Testing the significance of the difference in the change: t					1.07	

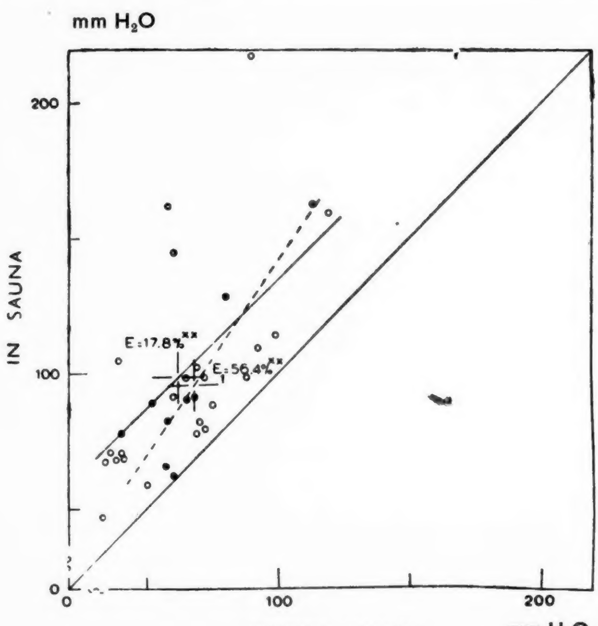

Fig. 8. — Graph showing individual determinations of the venous blood pressure before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant increase in the venous blood pressure; regression lines reveal significant correlation of the venous blood pressure in the sauna on that before the sauna.

O = healthy subjects, ● = hypertensive subjects, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

showed a clear tendency to rise in the sauna (Table VIII). Before the sauna the venous blood pressure varied between 40 and 113 mmH_2O. The mean rise in the hypertensive subjects was 28 mmH_2O, 9 mmH_2O lower than in the healthy subjects. The difference in the rise in the two groups was not statistically significant. The rise in the venous blood pressure of the hypertensive patients was statistically significant, and a significant correlation was observed to exist between the venous blood pressure before and during the sauna (Fig. 8).

PULSE RATE

The increase of the pulse rate in the sauna in the group of healthy subjects was 61% compared with the starting value (Table IX).

TABLE IX

PULSE RATE PER MIN. IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	51—80	60—90	79—137	82—167	+13— +69	+18— +77
Mean	64	72	104	119	+40	+47
Standard error of the mean	1.5	3.4	3.1	9.5	3.0	8.2
Percentage change from values before sauna					+61	+65
Testing the significance of the change: t					13.11***	5.72***
Testing the significance of the difference in the change: t					0.85	

In the healthy subjects the maximum and minimum pulse rates in the sauna were 137/min. and 79/min. respectively. The increase in the pulse rate during the sauna compared with the values before the sauna was highly significant statistically. The individual pulse rates before and during the sauna are shown in Fig. 9. No correlation could be observed between the pulse rate values before and during the sauna. Nor was there any correlation between the rectal temperature and the pulse rate (Fig. 10).

The pulse was regular and uniform before and during the sauna in all cases.

The increase in the pulse rate in the sauna was approximately

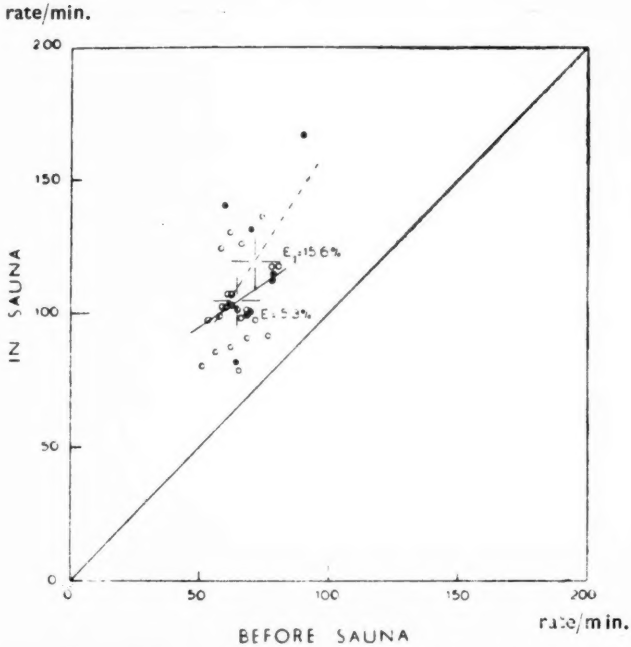

Fig. 9. — **Graph showing individual determinations of the pulse rate before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant increase in the pulse rate; regression lines show no correlation of the pulse rate in the sauna on that before the sauna.**

O = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

equal in both groups (65% to 61%, Table IX). The difference in the change of the pulse rate between healthy subjects and hypertensive patients was not statistically significant. The hypertensive patients also showed a regular and uniform pulse rate in the sauna. The maximum pulse rate among the hypertensive patients was well above the corresponding value for the healthy subjects (167/min., 137/min.). The mean pulse rate in the sauna was 119/min. The increase in the pulse rate was highly significant statistically. No correlation could be observed between the pulse rate values obtained before and during the sauna (Fig. 9).

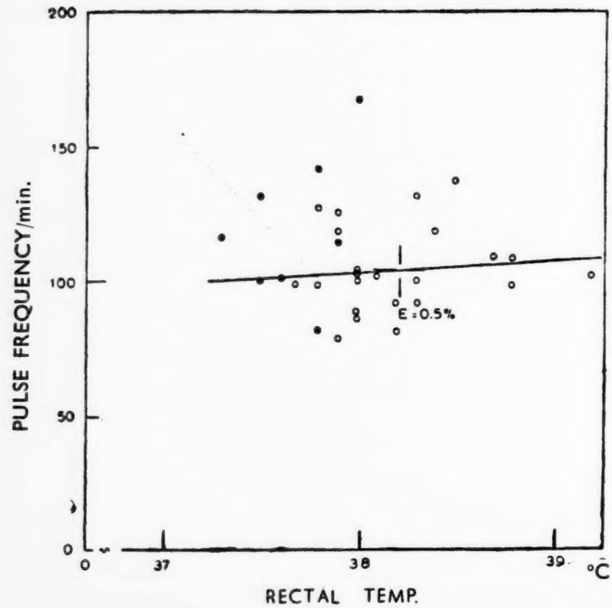

Fig. 10. — **Graph showing the correlation of the pulse rate frequency and the rectal temperature (final determination in the sauna), together with a regression line expressing the average relation for healthy subjects. Regression line reveals no correlation between the pulse frequency and rectal temperature.**

O = healthy subjects, ● = hypertensive patients, + = average for healthy subjects, — = regression line for healthy subjects, E = efficiency percentage for healthy subjects.

MEAN CIRCULATION TIME

During the sauna there was a distinct change in the mean circulation time from the cubital vein in one arm to the brachial artery in the other arm in healthy subjects. The circulation time decreased on the average by 59% compared with the values obtained before the sauna (Table X).

The circulation times taken before the sauna varied considerably, from 12.9 to 32.5 sec., the mean time being 21.0 sec. This agrees with the results of Eliasch (1952) who observed marked variations in the mean circulation time of normal subjects. Blumgart *et al.* (1927) reported that the circulation time from one arm to the other was between 14 and 24 sec. Hamilton *et al.* (1932 and 1948) reported a mean circulation time of 20 sec. Eliasch (1952) and Doyle *et al.* (1953) determined the circulation time from the pul-

TABLE X

MEAN CIRCULATION TIME (SEC.) FROM ONE ARM TO THE OTHER IN HEALTHY SUBJETS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	12.9— 32.5	12.4— 27.8	5.3— 17.2	5.2— 12.3	-24.5— +1.0	-21.0— -3.2
Mean	21.0	18.2	8.7	8.5	-12.3	-9.7
Standard error of the mean	1.2	2.1	0.5	0.8	1.3	2.0
Percentage change from values before sauna					-59	-53
Testing the significance of the change: t					9.61***	4.96**
Testing the significance of the difference in the change: t..					1.12	

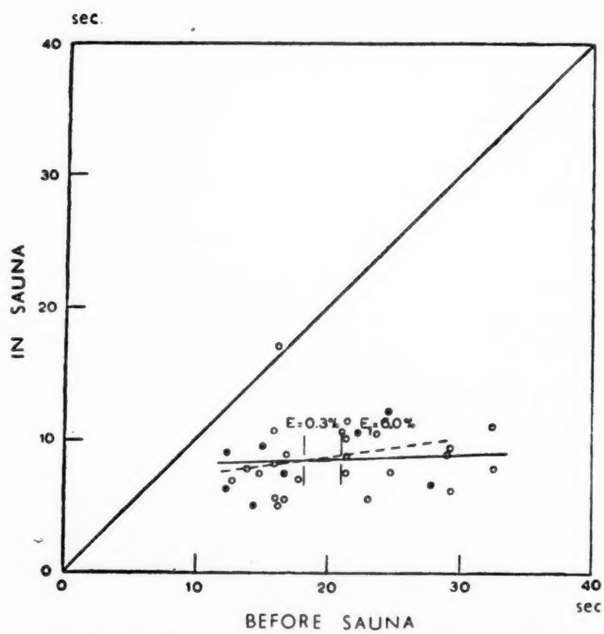

Fig. 11. — Graph showing individual determinations of the mean circulation time before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant decrease in the mean circulation time; regression lines reveal no correlation of the mean circulation time in the sauna on that before the sauna.

O = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

monary artery to the brachial artery and reported a mean circulation time of c. 11 sec.

The mean decrease in the circulation time in the sauna was 12.3 sec. This change was highly significant statistically. The relationship between the mean circulation times of each subject before and during the sauna is shown in Fig. 11. No correlation could be observed between the mean circulation times before and during the sauna.

The mean circulation time before the sauna in hypertensive patients was 18.2 sec. In the sauna the circulation time decreased in all subjects tested (Table X), the decrease being 53%. The mean circulation time in the sauna was 8.5 sec. for the hypertensive patients compared with 8.7 sec. for normal subjects. The decrease in the circulation time was significant. No statistically significant difference between the hypertensive patients and the healthy subjects could be observed in the change in the circulation time during the sauna. Fig. 11 shows the change in the mean circulation time before and during the sauna in the individual cases. There was no correlation between the values obtained before and in the sauna.

CARDIAC OUTPUT AND CARDIAC INDEX

The cardiac output of healthy subjects showed a highly significant increase in the sauna (Table XI).

The cardiac output before the sauna ranged between 3.06 and 7.76 litres per min., the mean being 5.31 l./min. The average in-

TABLE XI

CARDIAC OUTPUT (LITRES PER MIN.) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	3.06—7.76	3.46—9.58	4.42—14.67	4.73—15.81	–0.36—+10.63	+1.27—+6.23
Mean	5.31	6.48	9.16	10.71	3.85	4.22
Standard error of the mean	0.23	0.73	0.65	1.19	0.64	0.53
Percentage change from values before sauna					+73	+65
Testing the significance of the change: t					6.01***	8.03***
Testing the significance of the difference in the change: t					0.44	

crease in the cardiac output in the sauna was 73%, the values varying between 4.42 and 14.67 l./min. In this latter case the time spent in the sauna was long (35 min.), and the wet bulb temperature was as high as +48°C. There was no distinct correlation between the cardiac output and the duration of the bath, the temperature of the sauna or the rectal temperature of the subject while in the sauna. No correlation could be shown between the values for the cardiac output before and during the sauna (Fig. 12). In two cases the cardiac output decreased in the sauna, one changing from 5.72 l. to 5.5 l. and the other from 7.76 l. to 7.4 l.

The changes in the cardiac index were obviously of the same magnitude as those of the cardiac output (Table XII).

TABLE XII

CARDIAC INDEX (LITRES PER MIN. PER SQ. METRE OF BODY SURFACE) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE SUBJECTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	1.65—	1.74—	2.36—	2.38—	-0.17—	+0.64—
	3.98	5.47	9.85	9.09	+6.52	+3.62
Mean	2.91	3.61	5.11	5.99	2.20	2.38
Standard error of the mean	0.13	0.42	0.39	0.70	0.39	0.30
Percentage change from values before sauna					+76	+66
Testing the significance of the change: t					5.68***	7.85***
Testing the significance of the difference in the change: t..					0.37	

No correlation could be shown between the values for the cardiac index before and during the sauna ($E = 2.9\%$), and, as already mentioned, there was no statistically significant correlation between the cardiac index and the temperature of the sauna, the duration of the bath, or the wet bulb temperature in the sauna.

The cardiac output in the hypertensive patients before the sauna varied between 3.46 l. and 9.58 l. (Table XI), the mean value being 6.48 l. The mean value in the sauna was 10.71 l., indicating an average increase in cardiac output of 65%. The cardiac output increased in all cases, the maximum increase being 6.23 l. and the mean 4.22 l. The subject with the highest cardiac output (15.81 l.) spent 18 minutes in the sauna; when the sample was taken the dry bulb temperature was +80°C, and the wet bulb temperature +38°C.

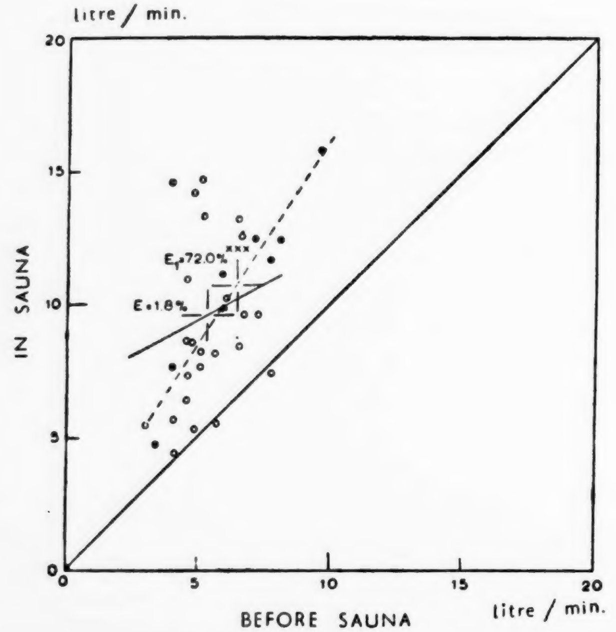

Fig. 12. — **Graph showing individual determinations of the cardiac output before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant increase in the cardiac output; regression lines reveal no correlation for healthy subjects and positive correlation for hypertensive patients of the cardiac output in the sauna on that before the sauna.**

O = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

The increase in the cardiac output of hypertensive patients in the sauna was highly significant statistically. A statistically highly significant correlation could be observed between the cardiac output before and during the sauna (Fig. 12). The difference between healthy subjects and hypertensive patients in the increase of the cardiac output in the sauna was not statistically significant.

Table XII shows that in the group of hypertensive patients the cardiac index increased by 66% in the sauna. The mean values for the cardiac index were 3.61 l./min./sq.m. before the sauna and 5.99 l./min./sq.m. in the sauna. This increase is highly significant statistically. The difference between the increase in the cardiac index of healthy subjects and that of hypertensive patients was not

statistically significant. The correlation between the cardiac index values before and during the sauna was highly significant ($E = 75.7\%^{***}$).

STROKE VOLUME

The changes in the stroke volume of healthy subjects in the sauna had no regular direction (Table XIII).

TABLE XIII

STROKE VOLUME (ML) IN HEALTHY SUBJECTS AND HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	45—117	51—121	42—136	47—136	-50— +53	-30— +30
Mean	83	88	88	92	5	4
Standard error of the mean	3.8	9.8	5.5	10.5	6.1	6.5
Percentage change from values before sauna					+6	+4
Testing the significance of the change: t					0.86	0.54
Testing the significance of the difference in the change: t					0.19	

The mean stroke volume was 83 ml. in the healthy subjects before the sauna. Grollman (1935) reported that the stroke volume of normal human subjects averages 62 ml, but considerable variations occur (Sodeman 1950, White 1951) and the stroke volume may be unusually high in young subjects, especially in athletes. The values in this investigation ranged between 45 ml. and 117 ml. The mean stroke volume was 88 ml. in the sauna, giving a mean increase of 6% for the stroke volume of healthy subjects. This change was not statistically significant. The highest value for the stroke volume in the sauna was 136 ml. No correlation could be shown between the values before and during the sauna (Fig. 13).

In the hypertensive patients the stroke volume varied between 51 ml. and 121 ml., with a mean value of 88 ml. (Table XIII). Changes of the stroke volume in both directions were also observed in hypertensive patients in the sauna, the mean change being a rise of 4%, which was not statistically significant. The maximum value recorded in the sauna was exactly the same as that for the healthy group,

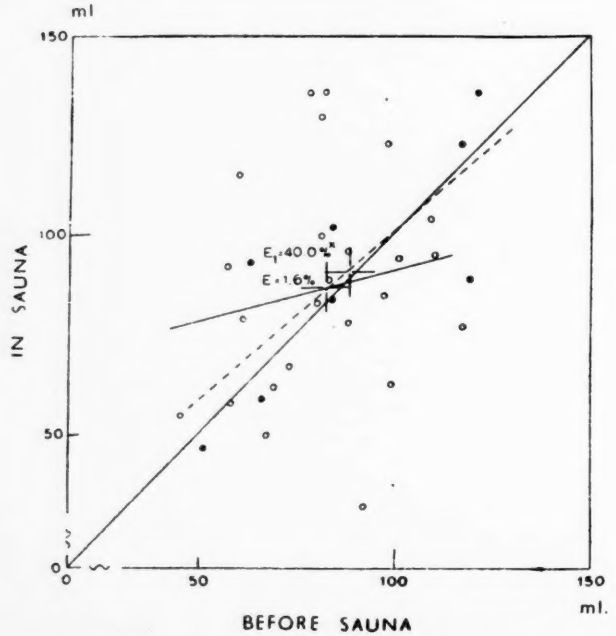

Fig. 13. — Graph showing individual determinations of the stroke volume before the sauna and in the sauna, together with regression lines expressing the average relations for each group. No significant change in the stroke volume; regression lines reveal no correlation for healthy subjects and positive correlation for hypertensive patients of the stroke volume in the sauna on that before the sauna.

○ = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

136 ml. An almost significant correlation could be shown between the values obtained before and those obtained during the sauna (Fig. 13). The difference between healthy subjects and hypertensive patients in the change of the stroke volume was not statistically significant.

CENTRAL BLOOD VOLUMES

The central blood volume was measured with the aid of two different methods and expressed in proportion to the surface area of the body (Tables XIV and XV).

The »from needle to needle»/BS before the sauna varied in healthy subjects between 0.53 and 1.42 l/sq.m with an average of 0.96 l/sq.m

TABLE XIV

FROM NEEDLE TO NEEDLE (LITRES PER SQ.METRE OF BODY SURFACE) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	0.53— 1.42	0.71— 1.29	0.33— 1.48	0.49— 1.02	–0.79— +0.54	–0.60— +0.54
Mean	0.96	1.01	0.71	0.81	–0.24	–0.20
Standard error of the mean	0.046	0.069	0.051	0.082	0.064	0.085
Percentage change from values before sauna					–25	–20
Testing the significance of the change: t					8.80***	2.36
Testing the significance of the difference in the change: t..					0.41	

TABLE XV

CENTRAL BLOOD VOLUME (LITRES PER SQ.METRE OF BODY SURFACE) IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	0.24— 0.74	0.29— 0.63	0.11— 0.91	0.15— 0.59	–0.42— +0.54	–0.40— +0.17
Mean	0.43	0.46	0.32	0.38	–0.11	–0.08
Standard error of the mean	0.028	0.043	0.037	0.056	0.042	0.064
Percentage change from values before sauna					–26	–17
Testing the significance of the change: t					2.69*	1.22
Testing the significance of the difference in the change: t..					0.45	

Hamilton et al. (1932) injected Evans' blue into the cubital vein and samples were taken from the femoral artery; this method gave a range of 1500—2000 cm^3/sq.m for the from needle to needle blood volume. Ebert *et al.* (1949) estimated the from needle to needle blood volume to be 1045$\pm$200 cm^3/sq.m. In the sauna the direction of the changes in the from needle to needle volume varied greatly (Fig. 14), but the blood volume between the needles was on the average 25% smaller than before the sauna. This change in the healthy subjects was highly significant. No correlation could be observed between the values obtained before and during the sauna (Fig. 14).

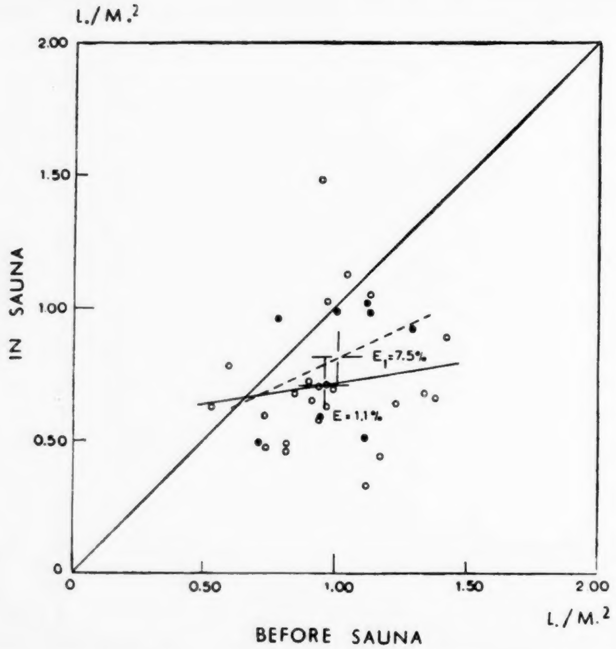

Fig. 14. — **Graph showing individual determinations of the from needle to needle volume before the sauna and in the sauna, together with regression lines expressing the average relations for each group. Significant decrease in the from needle to needle volume in the healtly subjects and no significant change in the hypertensive patients; regression lines reveal no correlation of the from needle to needle volume in the sauna on that before the sauna.**

O = healthy subjects, ● = hypertensive patients, + = averages, — = regression line for healthy subjects, - - - = regression line for hypertensive patients, E = efficiency percentage for healthy subjects, E_1 = efficiency percentage for hypertensive patients.

The central blood volume measured by Newman's method decreased by 26% in healthy subjects in the sauna. This change was almost significant statistically. No correlation between the values before and in the sauna could be observed ($E = 2.0\%$). The correlation between the values obtained by the two different methods was highly significant statistically ($E = 54.4\%$***).

The from needle to needle blood volume of the hypertensive patients before the sauna varied from 0.71 to 1.29 l./sq.m., with an average of 1.01 l./sq.m. (Table XIV). The changes in the sauna varied between —0.60 and +0.54 l./sq.m., but gave a mean decrease of 20%. No correlation could be shown between the from needle to needle values obtained before and in the sauna (Fig. 14), nor was the

difference in the changes of the from needle to needle blood volume between the healthy and the hypertensive subjects statistically significant.

A mean decrease in the central blood volume measured by Newman's method was also observed in the hypertensive subjects in the sauna. The decrease was 17% (Table XV), the mean values for the central blood volume being 0.46 l./sq.m. before the sauna and 0.38 l./sq.m. during the sauna. Changes could be observed in both directions varying between +0.17 and —0.40 l./sq.m. The change was not statistically significant. No correlation could be observed between the values before and during the sauna (E = 1.8%), and the difference in the changes of the central blood volume between healthy and hypertensive subjects was not significant.

BODY WEIGHT

The body weight of all test subjects decreased in the sauna (Table XVI).

The decrease in the body weight of healthy subjects varied between 2.1 and 0.2 kg. The mean decrease was 1.3%, statistically a highly significant change.

In the hypertensive subjects the decrease in body weight was slightly smaller, with an average of 0.7 kg (Table XVI).

TABLE XVI

BODY WEIGHT (kg) IN HEALTHY AND IN HYPERTENSIVE SUBJECTS BEFORE AND DURING THE SAUNA

	Before Sauna		In Sauna		Change	
	Healthy	HS	Healthy	HS	Healthy	HS
Range	55.0— 92.8	53.7— 82.7	52.9— 92.2	53.1— 82.4	-2.1— -0.2	-0.9— -0.3
Mean	70.4	70.9	69.4	70.2	-0.9	-0.7
Standard error of the mean	2.0	3.5	2.1	3.5	0.1	0.1
Percentage change from values before sauna					-1.3	-0.9
Testing the significance of the change: t					7.58***	7.53***
Testing the significance of the difference in the change: t..					1.68	

GENERAL CHART FOR CHANGES IN HEALTHY SUBJECTS

Figure 15, which gives a general view of the results, is plotted on a logarithmic scale. The scale factor has been kept constant, so

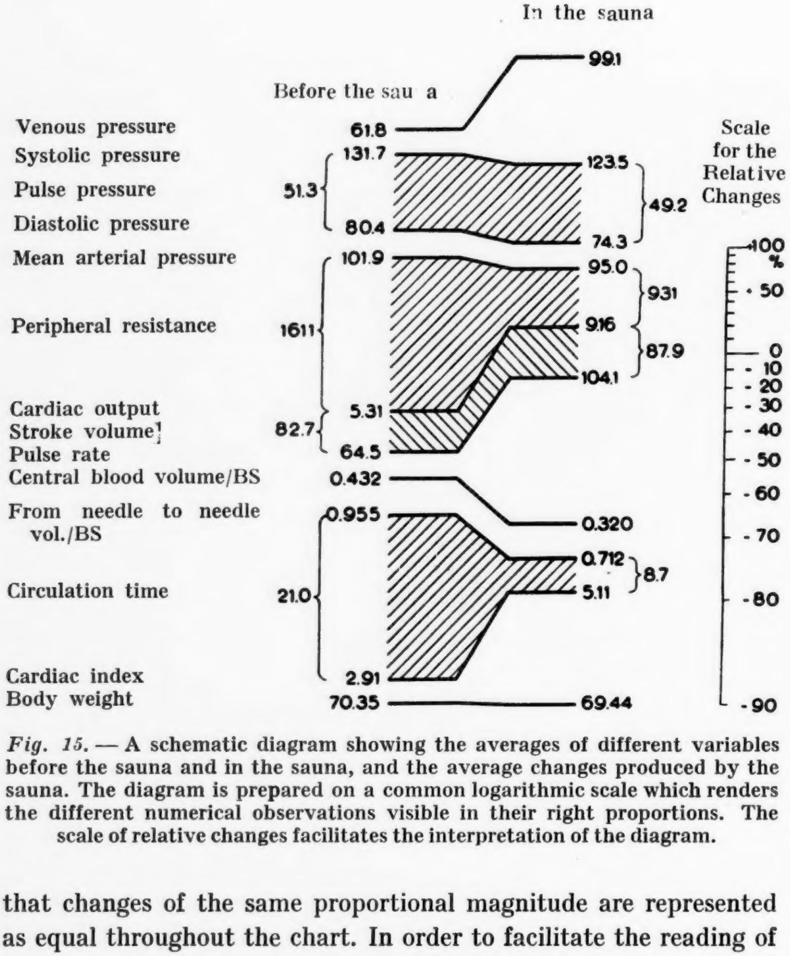

Fig. 15. — A schematic diagram showing the averages of different variables before the sauna and in the sauna, and the average changes produced by the sauna. The diagram is prepared on a common logarithmic scale which renders the different numerical observations visible in their right proportions. The scale of relative changes facilitates the interpretation of the diagram.

that changes of the same proportional magnitude are represented as equal throughout the chart. In order to facilitate the reading of the chart, a scale indicating the size of the relative changes has been added. This scale can be used for any part of the figure.

The logarithmic scale is convenient in presenting quotients, for any quotient may be shown as the distance between the dividend and the divisor. In this way it is possible to represent the changes in such quotients as shaded areas. When, e.g., the peripheral resistance is determined by dividing the mean arterial blood pressure by the cardiac output (and by multiplying this quotient by a constant), the change in the distance between the pressure and the cardiac output curves indicates the relative magnitude of the change in the resistance. It must be observed that the distances on the scale

are arbitrary, and in estimating the relative changes we have to note the size of the change in the distance, not the actual extent of the distance.

Because of its peculiarity the case of V. S. will be described in detail. This subject, who was very accustomed to hot baths, stayed in the sauna for 80 min. While in the sauna he splashed cold water onto his body. The mean temperature of the sauna was + 76° C, the wet bulb temperature + 46° C, and the subject lost 3.1 kg in body weight. After the sauna his skin temperature was 38.2° C and his rectal temperature 37.4° C. Some of the values were taken in the dressing room 10 minutes after the bath.

No changes were observed in the mean arterial, diastolic and systolic blood pressures, which remained at 92, 80 and 108 mmHg respectively. The venous blood pressure rose by + 94% (from 50 to 97 mmH_2O) whereas the mean rise for the other healthy subjects was + 60%. The increase in the pulse rate was + 20% (from 54/min to 65/min) compared with 61% in the other subjects. The cardiac output increased from 6.17 to 6.83 l/min. or 11% (average for the others 73%). The circulation time decreased by 40% (from 32 to 19.2 sec.) compared with — 59% in the others; the peripheral resistance fell from 1189 to 1074 units (— 9% compared with an average of —42% for the others) and the stroke volume declined from 114 to 105 ml. The »from needle to needle» blood volume fell from 1.70 to 1.15 l/sq.m.

Although the experimental conditions were not exactly the same as for the other tests, this special case does serve to show the effect which training may have upon the various physiological changes produced by the sauna.

DISCUSSION

EXPERIMENTAL CONDITIONS

The temperature and the humidity of typical Finnish saunas vary to a certain extent, and no attempt has been made in this investigation to arrange exactly similar experimental conditions for each subject. It would, in fact, have been impossible to use a set temperature owing to the differences in the subjects' ability to withstand heat (Lampert 1955). The subjective heat tolerance of each subject was taken as a heat regulator, so that each subject bathed at the sauna temperature to which he was accustomed. The tests were effected when the subject felt that he had bathed long enough. The experimental results, thus, give a picture of the hemodynamic changes produced by a normal Finnish sauna.

EFFECT OF THE SAUNA ON HEALTHY SUBJECTS

The pulse rate increased by 61% in the sauna, the mean pulse rate in the sauna being 104/min. All previous investigators reported an increase in the pulse rate in the sauna (Lundgren 1933, Fey 1942, v. Knorre 1943, Ott 1948, Räsänen 1951, Eggers and Goll 1952, Klingler-Mandig 1952, Schröder and Eckhardt 1952, Prokop 1953, Hasan and Niemi 1954, Luder 1954).

The changes in the *blood pressure* in the sauna were slight. The mean arterial blood pressure was 102 mmHg before and 95 mmHg during the sauna. Individual changes in the arterial blood pressure varied greatly, the maximum fall being 32 mmHg and the maximum rise 4 mmHg. Although the fall in the mean arterial blood pressure in the sauna was highly significant statistically, its magnitude was probably too small to be of any great practical importance. Many different factors have an effect upon the mean arterial blood

pressure. An increase in the cardiac output will cause it to rise (Wiggers 1949), but the sauna also causes a decrease in the peripheral resistance and this will produce a fall in the mean arterial blood pressure. The opposing effects of these factors explain the comparatively small changes which occur in the mean arterial blood pressure in the sauna.

The *systolic blood pressure* showed a slight change in the sauna, the mean fall being 8 mmHg, the maximum fall 50 mmHg and the maximum rise 24 mmHg. The mean change was so small, that the systolic blood pressure can be considered as practically unchanged in the sauna. Some other investigators have reported similar results e.g. Klingler-Mandig (1952), Prokop (1953) and Covalt (1954). On the other hand, some workers have reported a slight increase, ranging between 10 and 20 mmHg, in the systolic blood pressure during the sauna (Lundgren 1933, v. Knorre 1943, Ott 1948, Devrient 1950, Eggers and Goll 1952 and Schröder 1952). Schröder and Eckhardt (1952) reported an increase of 35—45 mmHg.

The following investigators reported an increase in the systolic blood pressure in a warm environment: Müller 1902, Adolph and Fulton 1924, Wiggers and Orias 1932, Gernandt *et al.* 1944, Proskauer *et al.* 1945, Glickman *et al.* 1947, Rodbard *et al.* 1950, Ederström 1954. Others, (Cheer 1928, Grollman 1930, Bierman and Fishberg 1934, Gottlieb 1935, Hyndman and Walkin 1941, Miller and Moor 1947, Wakim *et al.* 1948, Prec *et al.* 1949, Olmstedt *et al.* 1951) observed great individual variations in a warm environment, but the mean value changed little. Bazett (1931) Sayers and Harrington (1921) and Glaser (1950) reported a decrease in the systolic blood pressure in a warm environment.

Varying results have been reported for the changes in the systolic blood pressure during warm water baths. According to Davies and Holmes (1930), Peemöller and Lund (1935) and Schad (1939) the systolic pressure will not rise in a water bath in which the temperature exceeds +37°C, but Strassburger (1905) observed a rise in the systolic pressure when the temperature of the water exceeded +40°C. Bazett (1924) observed considerable individual variations and Tigerstedt (1917) reported a rise of 26% in the systolic pressure of anaesthetized rabbits. Grefberg (1882) reported an increase of up to 38 mmHg in the systolic pressure of anaesthetized rabbits when exposed to a water bath of +40°C. Schliephake *et al.* (1953),

on the other hand, reported a decrease of the systolic blood pressure immediately after a warm bath of +37°C.

A fall in blood pressure has been observed in hypothermal experiments with animals. In anaesthetized cats and dogs a mean arterial blood pressure of 128 mmHg at +38°C fell to 116 mmHg when their body temperature was lowered to +34°C. When the animals were kept in the cold environment until their body temperature had decreased to +18°C, the mean arterial blood pressure fell to 31 mmHg (Hook and Stormont 1941). Prec *et al.* (1949) observed a fall of 54% in anaesthetized dogs when the body temperature was lowered to +29°C. Bigelow *et al.* (1950) reported a fall in the mean arterial blood pressure from normal values of 110—130 mmHg to values of 16—46 mmHg at a body temperature of +20°C. Wayburn (1947) took the blood pressure of 6 human subjects after they had been in cold water for 20—35 min., and their rectal temperature was +35°C. The systolic blood pressure decreased below 100 mmHg in three cases, a slight decrease was observed in two subjects and there was no noticeable change in one subject. The diastolic blood pressure decreased in all 6 cases.

Individual variations similar to those in the systolic pressure were observed in the *diastolic blood pressure* in the sauna. The maximum fall in the diastolic blood pressure was 20 mmHg, and the maximum rise 15 mmHg, the average being a decrease of 6 mmHg. This change seems to have no more practical significance than the change in the systolic pressure. Fey (1942), Bardenheuer (1943), v. Knorre (1943), Gernandt *et al.* (1944), Karsten (1948), Devrient (1950), Eggers and Goll (1952), Schröder (1952), and Prokop (1953) obtained similar results in their studies of the changes in diastolic blood pressure induced by the sauna. Bartels (1944) and Luder (1954), on the other hand, reported a rise in the diastolic blood pressure in the sauna.

However, a slight decrease in the diastolic blood pressure in the sauna has been observed in most previous investigations.

The diastolic pressure has been shown to decrease in an environment of hot air (Sayers and Harrington 1921, Adolph and Fulton 1924, Bierman and Fishberg 1934, Hyndman and Walkin 1941, Wezler and Thauer 1943, Glickman *et al.* 1947, Miller and Moor 1947, Wakim *et al.* 1948 and Threefoot 1952). Wiggers and Orias (1932), Gottlieb (1935) and Rodbard *et al.* (1950), however, reported results in the opposite direction. Madson (1940) reported that a decrease in both the systolic and the diastolic blood pressures generally occurs when people move from a colder climate to the tropics.

Peemöller (1935), Davies and Holmes (1930), Herkel (1939), Kunze (1940), Schliephake *et al.* (1953) reported decreases in the diastolic blood pressure in warm water baths. Bornstein *et al.* (1931) and Schad (1939) observed no change in the diastolic pressure in warm water baths.

The slight changes in the systolic and the diastolic pressures in the sauna meant that the *pulse pressure* changes were small. The mean fall in the pulse pressure in the sauna was 2 mmHg, changes in individual cases varying between —50 mmHg and +34 mmHg. Bartels (1944), Räsänen (1951), Klingler-Mandig (1952), Prokop (1953) reported similar changes, whereas Fey (1942), Bardenheuer (1943), v. Knorre (1943), Karsten (1948), Devrient (1950), Eggers and Goll (1952), Schröder (1952) and Luder (1954) observed increases in the pulse pressure in the sauna.

The venous blood pressure rose in the sauna by an average of 60%, but the increase varied greatly in individual cases. The maximal venous blood pressure measured in the sauna was 218 mmH_2O. The mean venous blood pressure in the sauna was within the normal range (Storstein 1949). The results correspond closely to other results obtained in the sauna (Klingler-Mandig 1952), and also to results obtained in hot environments (Henry and Gauer 1950, Threefoot (1952). One of the reasons for the increased venous blood pressure in the sauna is the dilatation of the dermal blood vessels and the opening of the arterio-venous shunts (Henry and Gauer 1950).

A rise in the venous blood pressure has also been observed in warm water baths. Schott (1922) reported a rise of 45—76 mmH_2O. Budelman (1933, 1934) observed a rise of up to 150 mmH_2O in the venous blood pressure of healthy subjects when exposed to hot water, but during the bath the values levelled off to 20—30 mmH_2O above the starting value. In cases with compensated cardiac disease the rise of the venous blood pressure was considerably higher than in normal subjects.

Apart from a rise in the venous blood pressure in the sauna and in warm water baths, it has also been observed that hot water and warm air will affect the circulation in different ways. In a water bath the intrapleural pressure will rise and this rise depends on the level of the water in the chest region. It has also been observed that the venous blood pressure in the extra-

thoracic veins depends exclusively upon the intrapleural pressure (Krüger and Budelman 1935).

The difference in the effect of warm water baths and warm air upon the circulation is due to the greater heat conductivity of water. In addition, hot water probably has a different sensory effect upon the skin (Grollman 1930), and the surrounding hot water has a hydrostatic effect upon the extrathoracic veins (Tigerstedt 1917). However, a hot air environment and a hot water environment both increase the body temperature. The response of the organism to this increase in temperature is an effort to increase the heat loss.

The *mean circulation time* showed a marked decrease in the sauna, the circulation time from one arm to the other being 59% less. The velocity of flow in the pulmonary region, thus, showed a clear increase.

The circulation time also decreases in warm water baths. Ude (1932) and Tietze (1937) observed decreases of up to 50% in such circumstances.

The increase in *the cardiac output* in the sauna was 73% of the starting value, the maximum value being 14.67 l. The changes observed correspond closely to those noted by Gernandt *et al.* (1944).

Barcroft and Marshall Jr. (1923), Marschak (1929), Grollman (1930), Ude (1932), Eismeyer and Czyrnick (1934), Wezler and Thauer (1943), and Wyndham (1951) observed an increase in the cardiac output in warm and hot environments.

In this investigation a decrease in the cardiac output was observed in only two cases. In one of these cases the decrease was from 5.72 l. to 5.50 l., an insignificant change almost within the range of methodic errors. The pulse rate in this case was considerably higher than the average (131/min.), the venous blood pressure increased to 162 mmH_2O, the central blood volume rose, and the mean circulation time increased by 1 sec. Some of these factors are considered as signs of accumulation of blood in the lungs (Hamilton *et al.* 1949) but it is hardly possible for this to be a case with cardiac decompensation. In the other case in which no increase of the cardiac output was observed, the subject was very tense at the beginning of the experiment, the systolic blood pressure and the pulse rate being high before he entered the sauna. In the sauna he calmed down considerably. The fall in his systolic blood pressure was the most marked in the group of healthy subjects.

Prec *et al.* (1949) and Wiggers (1949) reported a decrease in the cardiac output in a hot environment. According to these investigators the venous return to the heart is decreased owing to the increased circulation of the skin and the dermal vasodilatation; this produces a decrease in the cardiac output.

The decrease in the cardiac output which occurs in hypothermia is also considered to be due to changes in the peripheral blood flow. Prec *et al.* (1949) reported that a decrease in the circulating blood volume and a retarded venous return will gradually cause a decrease in cardiac output. They observed a decrease of 35—56% in the cardiac output of anaesthetized dogs when their body temperature was lowered to +29°C. Bigelow *et al.* (1950) reported that the cardiac output in anaesthetized dogs was 14% below the starting value at a body temperature of +20°C.

Bornstein (1910) found no changes in the cardiac output when his subjects were exposed to a water bath with a temperature of +32°C, but he observed a considerable increase in the cardiac output at temperatures of +37°C—+40°C. Schapels (1912) reported a slight increase at these temperatures. Tigerstedt (1917) determined the cardiac output in anaesthetized rabbits; using a »Stromuhr» he observed that when the temperature of the water rose from +35°C to +46°C the cardiac output increased from 3 to 47%. Bornstein *et al.* (1931) using the nitrous oxide method observed an increase of 84% in the cardiac output when the temperature of the water was +33°C and at a temperature of +38.5°C the increases ranged between 114 and 329%. Budelman (1933, 1934) using Grollman's method observed that the cardiac output of healthy subjects was doubled at a water temperature of +38.5°C, but in patients with cardiac disease no change in the cardiac output was observed. Winterstein and Fraenkel-Tessman (1933) observed no increase in the cardiac output when healthy subjects were kept in a water bath with a temperature of +36°C—+37°C; in some cases the cardiac output decreased by 13—55%. Kroetz and Wachter (1933) using Grollman's method observed an increase in the cardiac output of healthy subjects ranging between 20 and 40% in a water bath of +35°C—+36°C. Voigt (1933) using the same method reported an increase of 46% when the subjects' hands and feet were kept in a bath of +45°C, and Eismeyer and Czyrnick (1934) also using Grollman's method observed an increase of 30—40% in a water bath of +42°C. Bazett *et al.* (1937) observed on increase in the cardiac index from 2.3

to 2.7. When Liljestrand and Zander's method was used an increase in the cardiac output of 25% was observed in healthy subjects in a water bath with a temperature of +38°C—+41°C (Hahn 1935). Kunze (1940) reported an increase of up to 700% when the temperature of the water bath rose from +35°C to +44°C. Herkel (1939), determining the cardiac output of healthy subjects by Wezler's and Böger's method, found that the increase of the cardiac output was 7—58% at a water temperature of +36°C, 50—89% at +39°C and 139% at +42°C.

The stroke volume showed no significant changes in the sauna, though large variations in both directions were observed in individual cases.

Grollman (1930) observed no marked change in the stroke volume in an environmental temperature increasing from 0°C to +45°C. Wezler and Thauer (1943) reported an increase in the stroke volume in a warm environment.

Different observations have been reported concerning the stroke volume changes in water baths with a temperature of +37°C —+44°C. Bornstein (1911) and Schapels (1912) reported a decrease, Hahn (1935) no change and Eismeyer and Czyrnick (1934) an increase in the stroke volume.

Due to an accelerated blood flow and to increased venous blood pressure, the venous blood return to the heart increased in the sauna. This could be regarded as one of the factors contributing to the increase in the cardiac output. No increase in the stroke volume occurred in the sauna, but the rise in the pulse rate was strong enough to cause a clear increase in the cardiac output.

Both the *from needle to needle volume* determined by Stewart's method and the *central blood volume* determined by Newman's method showed a decrease in the sauna. The decrease in the from needle to needle volume was highly significant statistically. No difference was observed between the changes in the from needle to needle blood volume and in the central blood volume in the sauna (t = 1.72). Care has to be shown in evaluating the significance of the change owing to the inaccuracy of the methods available for determining the central blood volume. Doyle *et al.* (1953) regarded changes of up to 30% as physiologically insignificant. Moreover, the relationship between the central blood volume and blood congestion in the lungs has not been classified. Hamilton *et al.* (1932)

observed an increase in the central blood volume of cases with cardiac failure. Kopelman and Lee (1951) showed that the intrathoracic blood volume, determined by the dye dilution method, increased during the decompensation phase in a case of left ventricular failure. It decreased during convalescence but remained slightly above normal. It was impossible to show a clear increase in the central blood volume of patients with mitral stenosis during the decompensation phase. According to Dastre-Morat's law the dermal vessels are dilated in a hot environment whereas the intestinal, lienal, renal and uterine vessels contract. The lungs are considered as being a storage place for blood as well as the splanchnic organs and Sjöstrand (1935, 1952) demonstrated by animal experiments that the lungs contain less blood in a warm environment than in a cold environment. Glaser *et al.* (1950) have shown by x-ray methods that the blood volume in the lungs and in the liver of human subjects decreases in a warm environment. An increase in the vital capacity is also regarded as a sign of a decrease in the blood volume in the lungs (Glaser 1949, Eggers and Goll 1952). Schröder (1952), Schröder and Eckhardt (1952) on the other hand showed that the vital capacity decreases after the sauna. However, any determination of the vital capacity in the sauna must be highly inaccurate due to the unpleasant effect which the breathing of hot air has upon the subject. Nor has the relation between the vital capacity and the blood volume of the lungs been fully explained (Asmussen and Nielsen 1955).

HYPERTENSIVE PATIENTS

The *pulse rate* of the hypertensive patients was on the average 65% higher, the maximum pulse rate in the sauna being 167/min. No difference could be observed between the rises in the pulse rate of the hypertensive patients and the healthy subjects.

The *blood pressure* changes in the hypertensive subjects in the sauna differed from those of the healthy subjects. In the hypertensive group the mean arterial blood pressure was 158 mmHg before and 137 mmHg in the sauna, the maximum fall being 32 mmHg. Moreover, the arterial blood pressure decreased in all hypertensive cases, whereas considerable variations in both directions could be observed in the healthy group.

All the hypertensive patients showed a fall in the *systolic blood pressure* in the sauna, the mean fall being 29 mmHg, the maximum 45 mmHg and the minimum 10 mmHg.

The *diastolic blood pressure* decreased in the sauna by an average of 14 mmHg. In two cases no change was observed.

The changes in the *pulse pressure* in the sauna were also similar in the hypertensive and healthy groups, the pulse pressure decreasing in all hypertensive cases as a result of the fall in the systolic pressure.

The fall in the systolic and diastolic blood pressures of the hypertensive patients which had begun in the sauna continued afterwards. 20 minutes after the sauna the mean systolic blood pressure was 151 mmHg compared with 205 mmHg before the sauna. Hence, the mean decrease was 54 mmHg. Although sources of error are known to exist in the determination of the blood pressure, especially in hypertensive subjects (van Bergen *et al.* 1954), the changes were quite significant. Thus, the sauna can be regarded as lowering the systolic and diastolic pressures of hypertensive patients.

The 20 hypertensive patients in whom only the blood pressure was determined also showed clear falls in the systolic and diastolic pressures in the sauna, the change in both pressures being highly significant statistically. One hour after the sauna the systolic pressure was on the average 35 mmHg less and the diastolic pressure 19 mmHg less than before the sauna. Any subjective symptoms such as headaches etc. had decreased or disappeared completely by the end of the sauna. In only one case a slight, temporary, stenocardiac pain was reported during the sauna. One subject (M.L.), had previously observed that after the sauna his headache and the stiffness of his tongue disappeared, this feeling of relief lasting c. 24 hours. Because of this he took a sauna regularly twice a week. Before the sauna on this occasion his speech was clearly stiff and he complained of a headache. After 10 minutes in the sauna he reported relief of the headache. His speech was also much easier up to one hour after the sauna.

The factors which have an effect on the arterial blood pressure are: 1) the pumping action of the heart, 2) the peripheral resistance, 3) the quantity of blood in the arterial system, 4) the viscosity of the blood, 5) the elasticity of the arterial walls (Best and Taylor 1945). The cardiac output increases in the sauna and this change

will increase the blood pressure. The viscosity of the blood and the elasticity of the arterial walls have not been investigated in this work; accordingly their effect upon the blood pressure changes in the sauna cannot be defined. Best and Taylor (1945) reported that in muscular exercise and fever the temperature of the blood rises, the viscosity of the blood is lowered and the work which the heart is called upon to do in overcoming the frictional resistance in the smaller vessels is thereby appreciably reduced. Hence, it may be that the sauna lowers the blood pressure by decreasing the viscosity of the blood. Changes in the quantity of blood in the arterial system in the sauna were not investigated. The plasma volume has been observed to increase in a hot environment but only after several days (Barbour 1921, Bazett 1927, 1938, Bazett *et al.* 1940, Conley and Nickerson 1945 and Strydom 1954). Glickman *et al.* (1941) reported an increase after several hours in a hot environment.

An increase in the elastic resistance, due to a decrease in the muscular tone of the arteries, has been observed in warm and hot environments (»der sichtbare Aussteig des elastischen Gesamtwiderstandes kann nur durch eine ausgiebige Erschaltung (Tonusverlust) der glatten Muskelelemente der grossen elastischen Arterien des Windkessels erklärt werden.» Wezler and Thauer 1943). This will cause a rise in the systolic blood pressure and a fall in the diastolic pressure. The decrease in the tone of the vessel will, however, increase its volume and, thus, with the increase in volume, the rise in the systolic pressure will be smaller.

The decrease in the *peripheral resistance* lessens both the systolic and the diastolic pressures. In healthy subjects the peripheral resistance decreased in the sauna by 680 units and in hypertensive patients by 1005 units. This difference was not significant ($t = 1.92$). Several investigators have reported a decrease in the peripheral resistance in a hot environment (Wezler and Thauer 1943, Adolph 1947, Wiggers 1949).

The lowering effect of the sauna on the blood pressure of hypertensive patients also has a practical significance. The uniform decrease in the hypertensive patients compared with the considerable variations in the healthy group indicates that the sauna definitely lowers the blood pressure of hypertensive patients, even though such factors as the lability of the systolic pressure and the possibilities of error in the measurements are taken into account.

Owing to our lack of knowledge of the etiology of hypertension it is difficult to determine all the factors which cause the lowering of the systolic and the diastolic pressures in and after the sauna. The aim of this work has been limited to investigating the relationship between the circulation and the sauna. It can however be mentioned that, according to present-day views, local or general vasoconstriction occurs in the arterioles in hypertension and, hence, the peripheral resistance is high. Consequently, the decrease in the peripheral resistance may be considered as one important factor in the decrease in the blood pressure of hypertensive patients during the sauna.

Crawford (1944) reported a decrease in the blood pressure of hypertensive patients when they moved to a tropical climate. Krauss (1953) did not regard hypertension as contraindicant to the sauna.

Kunze (1940) demonstrated a slight decrease or no change at all in the diastolic blood pressure of hypertensive patients exposed to warm baths. Herkel (1939) reported decreases similar to those in healthy subjects.

Page and Corcoran (1945) reported an increase in both the systolic and the diastolic blood pressures of hypertensive patients exposed to cold water.

The mean *venous blood pressure* of the hypertensive patients rose by 41% in the sauna. No diffrences could be observed between healthy subjects and hypertensive patients.

Nor could any difference between the two groups be observed in the changes in the *circulation time*. The mean circulation time of hypertensive patients decreased by 53% in the sauna.

The *cardiac output* of hypertensive patients increased in the sauna by 65%, a change of the same magnitude as in the healthy subjects. The maximum value for the cardiac output in the sauna, observed in a hypertensive patient, was 15.81 l.

The changes in the *stroke volume* in the sauna were also similar for both healthy subjects and for hypertensive patients. Considerable variations occurred in the individual cases, but the mean change was not significant.

A slight decrease was observed in the mean *central blood volume* of the hypertensive patients in the sauna. This change was smaller than that in healthy subjects, but the difference was not significant.

STABILITY OF THE INDIVIDUAL VARIATIONS IN THE SAUNA

On examining the results, we find that individual variations are a distinct feature of many of the determinations. Wezler and Thauer (1943) and Ott (1948) also observed marked individual differences in their investigations into hot environments. The following table shows, in terms of efficiency percentage (see p. 33). the correlations between the results obtained before and in the sauna.

	Healthy Subjects	Hypertensive Patients
Mean arterial blood pressure	E = 25.8%***	E = 26.7%
Diastolic blood pressure	E = 22.3%***	E = 11.2%
Systolic blood pressure	E = 10.7%*	E = 47.3%**
Pulse pressure	E = 3.7%	E = 56.4%**
Venous blood pressure	E = 17.8%**	E = 56.4%**
Pulse rate	E = 5.3%	E = 15.6%
Mean circulation time	E = 0.3%	E = 6.0%
From needle to needle volume/BS	E = 1.1%	E = 7.5%
Central blood volume/BS	E = 2.0%	E = 1.8%
Peripheral resistance	E = 12.8%*	E = 60.8%**
Cardiac output	E = 1.8%	E = 72.0%***
Stroke volume	E = 1.6%	E = 40.0%*

Thus, for many of the hemodynamic factors it is difficult to predict in individual cases the changes which will occur in the sauna, even if we know the pre-sauna values. All the factors which are not marked with an asterisk belong to this group. The significance of the individual differences has to be noted, if we are to get a true general impression of the effect of the sauna upon the circulation.

SUMMARY

The aim of this investigation has been to study the changes produced in the circulation by the Finnish sauna.

The following hemodynamic factors were determined in 25 healthy subjects and in 8 hypertensive patients before and in the sauna: the cardiac output, the stroke volume, the mean circulation time and the intrathoracic blood volume determined by the dye dilution, the central blood volume determined according to Newmann's principle, the pulse rate, the arterial blood pressure determined by a mercury manometer, the peripheral resistance determined by the indirect method and the venous blood pressure taken at the cubital vein.

In addition, the blood pressure changes in the sauna and for one hour after the sauna were followed in 20 hypertensive patients.

The cardiac output increased by an average of 73% in the healthy subjects and 65% in the hypertensive patients.

The stroke volume showed no systematical change in either group.

The mean circulation time decreased in both groups, being 59% less in the healthy subjects and 53% less in the hypertensive subjects.

The intrathoracic blood volume, determined by Stewart or by Newmann's methods, decreased in the sauna. No difference in the change could be observed between the healthy and the hypertensive group.

The increase in the pulse rate was 61% in the healthy subjects, and 65% in the hypertensive patients.

The systolic blood pressure decreased slightly in the healthy subjects but the change had no practical importance. In the hypertensive patients the mean decrease in the systolic pressure was 29 mmHg. 20 minutes after the sauna the mean decrease was

54 mmHg. In the 20 hypertensive patients in whom only the blood pressure was determined a mean decrease of 35 mmHg was observed in the systolic blood pressure one hour after sauna. In both groups of hypertensive patients the fall in the systolic blood pressure was highly significant statistically.

The diastolic blood pressure remained practically unchanged in the healthy subjects. In the hypertensive patients the diastolic pressure decreased. In the group of 8 patients with essential hypertension the mean decrease in the sauna was 14 mmHg and in the other test group the diastolic blood pressure one hour after the sauna was on average 19 mmHg less than the starting value. The changes in the diastolic blood pressure were statistically significant.

The peripheral resistance decreased in both healthy subjects and hypertensive patients. The changes in both groups were highly significant statistically.

The venous blood pressure of the healthy subjects was 60% higher in the sauna. In the hypertensive patients the increase was 41%.

The results obtained are compared with the results of earlier investigations performed in warm water baths.

APPENDIX

Appendix 1. Individual determinations for healthy subjects before (upper figure) the sauna and in (lower figure) the sauna.

Appendix 2. Individual determinations for hypertensive patients before (upper figure) the sauna and in (lower figure) the sauna.

Appendix 3. Individual blood pressure determinations for the hypertensive patients for whom the blood pressure only was measured.

APPENDIX N:o 1

Subjects	Cardiac Output, Litres	Cardiac Index, l./min./sq.m.	Stroke Volume, ml.	Mean Circulation Time, sec.	From Needle to Needle, litres	From Needle to Needle/BS	Central Blood Volume, litres	Central Blood Volume/BS	Pulse Rate/min.	Blood Pressure, mmHg
AT	5.20	3.33	81	16.8	1.46	0.94	0.57	0.37	64	128/80
	13.30	9.85	130	9.0	2.00	1.48	1.23	0.91	102	152/70
PS	5.12	2.77	97	29.1	2.48	1.34	0.79	0.43	53	138/90
	8.20	4.43	84	9.2	1.26	0.68	0.40	0.22	98	110/80
VR	6.05	2.94	101	14.9	1.50	0.75	0.79	0.38	60	160/90
	10.20	5.00	94	7.7	1.31	0.64	0.61	0.30	108	110/70
UP	6.57	3.98	109	21.5	2.35	1.42	1.02	0.62	51	110/70
	8.45	5.18	104	10.3	1.45	0.89	0.43	0.26	81	108/70
OS	6.65	3.45	98	16.3	1.81	0.94	1.15	0.60	68	128/75
	12.54	6.53	123	5.3	1.11	0.58	0.78	0.41	102	115/65
MA	4.82	2.74	78	24.8	1.99	1.13	1.08	0.61	62	125/75
	14.16	8.05	136	7.8	1.84	1.05	1.02	0.58	104	125/70
KH	3.98	2.21	60	16.1	1.07	0.59	0.48	0.27	66	160/85
	14.61	8.12	115	5.8	1.41	0.78	0.64	0.36	127	160/75
HL	7.76	3.66	99	15.9	2.06	0.97	0.73	0.34	78	158/90
	7.40	3.49	63	10.8	1.33	0.63	0.38	0.18	118	108/90
RP	5.09	2.51	82	23.1	1.96	0.97	1.07	0.53	62	120/75
	14.67	7.35	136	5.8	1.42	0.71	0.62	0.31	108	108/70
OG	6.51	3.58	88	16.7	1.81	0.99	0.94	0.52	74	134/70
	13.19	7.33	96	5.7	1.25	0.69	0.55	0.31	137	140/70
MK	3.06	1.65	45	29.3	1.49	0.81	0.64	0.35	68	168/104
	5.50	2.97	55	9.6	0.88	0.48	0.21	0.11	100	140/104
EK	7.24	3.87	110	21.4	2.58	1.38	0.94	0.50	66	132/90
	9.62	5.14	97	7.7	1.23	0.66	0.64	0.34	99	132/70
KS	4.58	2.65	57	23.6	1.80	1.04	1.28	0.74	80	118/65
	10.89	6.37	92	10.6	1.92	1.12	0.92	0.54	118	110/65

SUBJECTS

Mean Art. Press-ure, mmHg	Peri-pheral Resist-ance, dyn.sec. cm—5	Venous Press-ure, mmH_2O	Hema-tocrit	Dry Bulb Temper-ature, °C	Wet Bulb Temper-ature, °C	Time Spent in Sauna, min.	Skin Temper-ature, °C	Rectal Temper-ature, °C	Body Height cm.	Body Weight kg.
100.0	1540	39	43	84 86	43 45	22	40.1 40.3	36.7	152	59.6
104.4	627	69	43	86	46		40.1	39.2 39.3		58.8
								39.2		
110.2	1720	69	43	68 67	38 39	18	39.2 39.1	36.9	172	74.0
92.6	902	78	44	68	38		39.1	37.7 37.9		73.2
								37.8		
119.4	1577	65	42	80 80	44 45	30	40.3 40.4	37.0	188	81.5
86.8	680	99	45	82	45		40.3	38.6 38.8		79.6
								38.8		
86.8	1056	50	43	76 76	48 49	18	39.4 39.4	37.1	164	61.1
86.0	814	92	44	78	48		39.5	38.1 38.1		59.0
								38.2		
97.3	1169	50	44	64 64	45 47	23	39.3 39.4	36.9	174	79.8
86.0	548	145	44	65	47		39.6	38.1 38.1		78.6
								38.1		
96.0	1592	40	44	60 61	46 46	21	39.8 39.8	37.1	174	64.2
93.1	525	70	44	62	46		39.8	37.9 37.9		63.6
								38.0		
116.5	2339	70	45	58 60	45 44	15	38.1 38.2	37.0	171	70.0
110.7	606	82	45	61	45		38.2	37.7 37.7		69.7
								37.8		
118.6	1221	34	42	65 64	40 41	18	38.1 38.0	36.8	181.5	91.4
97.6	1054	68	42	65	41		38.1	37.8 37.8		90.9
								37.9		
93.9	1474	33	43	68 69	47 48	35	38.9 39.2	37.1	190	77.2
86.0	468	47	44	69	48		39.2	38.5 38.7		75.6
								38.7		
96.9	1190	90	43	80 82	47 48	15	41.7 41.6	36.9	170	71.7
99.4	603	218	43	82	48		41.6	38.4 38.4		70.0
								38.5		
130.9	3419	50	42	54 56	40 44	16	39.8 39.8	37.1	175	71.5
119.1	1730	59	42	57	45		39.9	37.9 37.9		71.1
								38.0		
107.6	1188	72	44	58 57	49 48	17	38.8 38.7	37.0	173	74.9
96.0	798	80	44	58	49		38.8	37.8 37.7		74.6
								37.7		
87.3	1523	92	45	72 76	40 41	18	40.7 40.2	36.8	172	62.2
83.9	616	110	44	75	41		40.1	37.1 38.2		61.1
								38.4		

Contin.

Subjects	Cardiac Output, Litres	Cardiac Index, l./min./sq.m.	Stroke Volume, ml.	Mean Circulation Time, sec.	From Needle to Needle, litres	From Needle to Needle/BS	Central Blood Volume, litres	Central Blood Volume/BS	Pulse Rate/min.	Blood Pressure, mmHg
AE	4.53	2.36	81	21.5	1.62	0.84	0.71	0.37	56	130/60
	8.63	4.52	100	9.0	1.29	0.68	0.39	0.20	86	125/75
AL	5.09	2.96	88	12.9	0.92	0.53	0.43	0.25	58	128/80
	7.75	4.51	78	7.1	1.09	0.63	0.34	0.20	100	125/75
EL	4.17	2.27	67	21.4	1.49	0.81	0.44	0.24	62	125/70
	4.42	2.42	50	11.5	0.85	0.46	0.30	0.16	88	123/70
RK	5.67	3.12	83	14.0	1.32	0.73	0.60	0.33	68	136/80
	8.12	4.46	89	8.0	1.08	0.59	0.48	0.26	91	136/75
TT	4.59	2.52	79	32.5	2.49	0.73	1.05	0.58	58	110/80
	6.40	3.56	62	8.0	0.85	0.47	0.29	0.16	103	105/60
IJ	4.84	2.16	73	32.3	2.61	1.17	1.01	0.45	66	132/80
	5.28	2.36	67	11.1	0.98	0.44	0.37	0.17	79	115/75
JJ	4.63	2.56	61	21.1	1.63	0.91	0.59	0.33	76	133/80
	7.31	4.04	79	10.7	1.30	0.72	0.43	0.24	92	128/70
KJ	5.72	3.60	92	16.2	1.54	0.97	0.63	0.40	62	108/70
	5.50	3.55	42	17.2	1.58	1.02	0.79	0.51	131	105/75
JL	4.09	2.28	58	29.3	2.00	1.12	0.95	0.53	71	115/85
	5.64	3.17	58	6.3	0.59	0.33	0.24	0.13	98	115/80
VS	6.17	3.20	114	32.0	3.29	1.70	0.78	0.40	54	108/80
	6.83	3.59	105	19.2	2.19	1.15	0.77	0.41	65	108/80
AV	4.82	3.07	80	17.8	1.43	0.91	0.39	0.25	60	125/95
	8.54	5.44	83	7.2	1.02	0.65	0.56	0.35	103	120/90
MJ	6.76	3.52	117	16.0	1.80	0.94	0.72	0.38	58	140/90
	9.62	5.04	77	8.3	1.33	0.70	0.92	0.48	125	150/70

Mean Art. Pressure, mmHg	Peripheral Resistance, dyn.sec. cm—5	Venous Pressure, mmH_2O	Hematocrit	Dry Bulb Temperature, °C	Wet Bulb Temperature, °C	Time Spent in Sauna, min.	Skin Temperature, °C	Rectal Temperature, °C	Body Height cm.	Body Weight kg.
89.4	1577	72	41	60 60	37 37	25	39.4 39.6	36.9	185	70.9
96.0	889	99	41.5	62	38		39.8	37.6 37.7		70.0
								38.0		
100.2	1573	49	41	68 70	37 38	17	39.6 39.4	37.1	176.5	58.9
96.0	990	103	41	72	40		39.4	38.2 38.3		58.3
								38.3		
93.1	1784	75	39	56 57	37 38	27	39.3 39.4	36.9	176	69.1
92.3	1669	89	40	59	38		39.4	37.8 37.8		68.7
								38.0		
103.5	1459	99	45	70 70	41 41	14	39.8 39.6	36.9	173	70.5
100.6	990	115	44.5	70	43		40.8	37.8 38.2		69.6
								38.3		
92.6	1612	119	41	68 69	41 43	19	38.6 38.6	37.1	176	67.2
78.9	985	160	42	71	44		38.8	37.7 37.9		66.0
								38.0		
101.8	1681	88	40	65 64	40 42	22	38.2 37.7	36.8	196	92.8
91.8	1390	99	41	65	42		38.4	38.2 38.2		92.2
								37.9		
102.3	1766	52	43	57 58	36 37	32	39.7 39.8	36.9	181.5	63.9
94.4	1032	89	43	58.5	39.5		39.7	39.2 38.2		63.6
								38.2		
86.0	1202	58	41	80 81	50 50	23	41.8 41.5	37.2	164	55.0
87.6	1873	162	42	83	51		41.3	37.9 37.9		52.9
								38.3		
97.6	1907	41	44	83 85	41 40	19	40.8 40.8	36.8	175	66.0
94.7	1342	69	43	83	41		40.9	38.5 38.6		65.6
								38.8		
91.8	1189	50	43	68 76	42 45.5	80	38.5 38.6	36.7	178	76.5
91.8	1074	97	45					37.9 37.9		73.4
107.6	1784	39	41	71 72	50 52	23	40.2 40.6	37.1	162	55.1
102.6	961	105	41	72	49		40.5	37.9 38.0		54.9
								38.0		
111.0	1312	36	41	63 63	47 48	18	39.1 39.1	36.9	172	80.0
103.6	861	71	41	62	50		39.2	38.0 38.0		79.1
								37.9		

APPENDIX N:o 2

Subjects	Cardiac Output, Litres,	Cardiac Index, l./min./sq.m.	Stroke Volume, ml.	Mean Circulation Time, sec.	From Needle to Needle, litres	From Needle to Needle/BS	Central Blood Volume, litres	Central Blood Volume/BS	Pulse Rate/min.	Blood Pressure, mmHg
KJ	3.46	1.74	51	24.5	1.41	0.71	0.57	0.29	68	246/120
	4.73	2.38	47	12.3	0.97	0.49	0.32	0.16	100	218/120
UK	5.90	3.04	84	22.1	2.17	1.12	0.99	0.51	70	230/130
	11.10	5.72	84	10.7	1.98	1.02	0.86	0.44	132	185/115
										150/90
OJ	7.70	3.99	84	15.1	1.94	1.00	1.22	0.63	78	175/115
	11.64	6.06	102	9.7	1.88	0.98	0.96	0.50	114	165/115
										110/60
AW	4.05	2.38	63	27.8	1.88	1.11	0.94	0.55	64	195/125
	7.64	4.49	93	6.8	0.87	0.51	0.25	0.15	82	158/105
										135/75
KH	7.16	3.87	119	14.5	1.73	0.94	0.71	0.38	60	195/110
	12.48	6.78	89	5.2	1.08	0.59	0.59	0.32	141	160/80
										150/90
CF	8.08	4.62	117	16.7	2.25	1.29	0.95	0.54	69	228/130
	12.40	7.17	123	7.7	1.59	0.92	0.65	0.38	101	190/105
										175/115
HA	9.58	5.47	121	12.4	1.98	1.13	0.78	0.44	78	210/110
	15.81	9.09	136	6.5	1.71	0.98	1.02	0.59	116	195/105
										175/100
AK	5.94	3.76	66	12.4	1.23	0.78	0.49	0.31	90	205/115
	9.86	6.24	59	9.2	1.52	0.96	0.76	0.48	167	180/100
										165/100

SUBJECTS

Mean Art. Pressure, mmHg	Peripheral Resistance, dyn.sec. cm−5	Venous Pressure, mmH_2O	Hematocrit	Dry Bulb Temperature, °C	Wet Bulb Temperature, °C	Time Spent in Sauna, min.	Skin Temperature, °C	Rectal Temperature, °C	Body Height cm.	Body Weight kg.
172.9	3994	68	43	55 56	40 40	12	38.0 37.9	36.9	180	80.9
161.2	2724	92	43	56	41		38.0	37.4 37.6		80.2
								37.5		
172.0	2330	61	43	57 57	41 44	15	38.0 38.4	37.1	171	82.7
144.4	1040	62	44	57	46		38.5	37.0 37.4		82.4
115.2								37.5		
140.2	1455	57	44	85 86	47 48	20	39.7 39.9	37.2	179	75.9
136.0	933	66	44	85	48		39.9	37.7 37.8		75.0
81.0								37.9		
154.4	3047	65	42	73 75	49 49	12	39.9 40.2	36.9	164	65.3
127.3	1331	91	42	75	50		40.1	37.3 37.6		65.0
100.2								37.8		
145.7	1626	40	44	77 77	39 42	14	40.2 40.2	37.1	167	77.2
113.6	727	78	44	77	43		40.0	37.2 37.6		76.4
115.2								37.8		
171.2	1693	80	44	76 78	40 43	10	39.6 40.0	36.9	170	64.8
140.7	907	129	44	78	44		39.8	37.2 37.2		64.0
140.2								37.6		
152.0	1268	113	43	78 80	34 38	18	38.2 38.4	37.1	170	66.3
142.8	722	163	43	80	38		38.4	37.1 37.2		65.4
131.5								37.3		
152.8	2056	58	43	74 75	41 42	23	40.1 41.4	36.9	165	53.7
129.4	1049	83	42	76	45		41.3	37.6 37.8		53.1
127.3								38.0		

APPENDIX N:o 3

HYPERTENSIVE SUBJECTS FOR WHOM THE

Subjects	Age, Years	Before Sauna, mmHg	In Sauna		
			5 min. mmHg	10 min. mmHg	15 min. mmHg
TK	48	205/115	210/110	180/105	205/100
VH	51	220/120	205/115	185/110	195/100
LK	50	235/130	180/115	185/115	145/95
MK	50	195/120	185/105	185/100	165/95
YS	55	160/110	175/120	175/120	165/110
AK	59	190/115	170/105	150/90	—
VK	63	235/110	215/100	215/100	220/105
KK	52	195/115	175/115	175/115	195/120
PP	49	190/120	150/105	145/95	145/95
VK	59	180/70	115/70	160/70	140/65
OV	54	205/80	205/85	170/75	205/80
ML	57	215/140	215/140	185/125	185/120
OH	63	220/110	205/100	205/95	205/95
TS	47	185/110	180/110	165/100	160/100
LH	43	210/115	170/110	165/105	155/100
AA	38	260/140	205/135	205/140	210/145
OV	44	230/130	170/115	170/105	175/100
OS	56	210/100	185/95	165/90	180/95
FM	56	230/140	230/135	230/130	200/100
RR	38	210/120	175/105	180/95	185/100

BLOOD PRESSURE ONLY WAS MEASURED

After Sauna			Dry Bulb Temperature/ Wet Bulb Temperature 5 min. °C°	Dry Bulb Temperature/ Wet Bulb Temperature at the End of Bath	Time Spent in Sauna, min.
20 min. mmHg	30 min. mmHg	60 min. mmHg			
165/85	180/100	205/105	69/38	70/39	15
165/100	170/100	175/105	70/40	69/41	15
165/110	155/100	165/100	67/40	69/40	15
160/95	160/95	160/95	69/40	69/40	15
130/90	130/95	155/95	80/32	79/40	15
145/95	160/95	155/95	78/32	78/35	12
190/100	195/115	200/100	74/31	72/31	15
175/110	175/115	190/110	84/34	82/34	15
165/105	145/105	150/105	78/34	77/34	15
150/70	135/65	150/70	92/32	89/32	15
190/50	175/60	180/60	86/32	79/32	15
190/130	155/110	180/130	72/31	72/31	15
190/100	190/100	220/115	80/36	80/32	15
155/100	150/100	145/95	80/36	80/36	15
150/100	160/100	155/100	80/34	82/34	15
205/140	195/140	195/140	89/34	90/34	15
180/115	185/110	180/115	90/34	88/34	15
140/80	150/90	160/95	79/34	82/34	15
210/135	175/115	180/105	79/41	89/41	15
180/100	175/115	175/110	87/39	87/39	15

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